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STUDIES ON THE B VIRUS. III: THE EXPERIMENTAL DISEASE IN *MACACUS RHESUS* MONKEYS.

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THE B Virus was isolated from a human case of ascending myelitis associated with visceral necrosis, which followed the bite of an apparently normal *Macacus rhesus* monkey (Sabin and Wright, 1934). Further studies on this virus (Sabin, 1934*a*, 1934*b*) revealed it to be a distinct entity, immunologically specific, with an apparent partial group relationship to the viruses of herpes simplex and pseudorabies (infectious bulbar paralysis, mad itch, Aujeszky's disease). Although peculiar vesiculo-pustular, necrotic lesions were present on the fingers, where they were bitten by the monkey, the relationship of the bite and the lesions to the ensuing human disease could not be proved. There were various possibilities: one was that the disease was coincidental and had no relationship to the bite; another that the bite might have given rise to the local lesion on the fingers, and that the virus causing the disease was a secondary invader; and finally, that the virus was introduced by the monkey-bite, and gave rise both to the local lesion and the systemic disease. Evidence to be presented in the present communication, taken together with other properties of the B Virus, suggests the last-named possibility as the most likely one. Unfortunately, the monkey died during an operation on the day of the bite, and was discarded. Attempts made in America to transmit the disease to *Macacus rhesus* monkeys with the human organs containing the virus were then considered to have been unsuccessful, but the present studies throw doubt on the correctness of that view. Aside from the information which it might yield with regard to the human disease, the investigation of the pathogenicity of the B Virus for rhesus monkeys was desirable also from the point of view of its relationship to pseudorabies and herpes simplex. While pseudorabies is distinctly pathogenic for rhesus monkeys (Hurst, 1933), all attempts to transmit herpes simplex to this species have been unsuccessful.

EFFECT OF COMBINED INTRACUTANEOUS AND INTRAPERITONEAL INOCULATION OF B VIRUS.

It was in the attempted production of an anti-B Virus serum that the monkeys were inoculated in the manner to be described, and the observations on the resulting lesions, although incidental at first, led to the realization that the virus was pathogenic for the *Macacus rhesus*.

Two monkeys were inoculated with 16th passage rabbit B Virus in the form of centrifuged brain and cord suspension. They were both full-grown, mature animals which had been at the Institute for several years. Monkey 2 had been injected intracerebrally about two years before, but remained entirely well. Monkey 3 had been used for poliomyelitis virus "neutralization" tests on various occasions in preceding years; five weeks before the present test it was inoculated intracerebrally with a large dose of poliomyelitis virus and remained entirely well, while other monkeys inoculated simultaneously with the same virus developed the disease and died.

Monkey 2 was first injected intracutaneously on the shaved abdominal skin with 1·0 c.c. (0·2 c.c. in 5 zones) of a 10 p.c. rabbit virus suspension (brain and cord). For the first two days there were only small white papules at the inoculated sites; on the 3rd day acutely inflamed red nodules about 1 cm. in diameter appeared at all the sites; on the 5th day they became necrotic, and several depressed vesicles containing purulent white matter appeared. At this stage the lesions looked remarkably like those seen on the fingers of the human case. At the end of one week, when the monkey appeared systemically entirely well, it was re-inoculated intracutaneously on the other side of the abdomen with 1·0 c.c., and intraperitoneally with 10·0 c.c. of a 10 p.c. virus suspension. Very slight œdema and erythema appeared at the newly injected sites on the second day, but instead of progressing to necrosis, faded rapidly. Thirteen days after the first inoculation the monkey was still entirely well.

It was then given a third inoculation of virus intracutaneously and intraperitoneally, the same virus suspension being used for a similar, but primary, inoculation of Monkey 3. Monkey 2 showed neither skin nor systemic reactions. Monkey 3, however, developed on the second day acutely inflamed nodules, about 1·0 cm. in diameter, at all the injected skin sites; on the 3rd day the lesions had increased in size to about 2·0 cm. in diameter, and central necrosis was apparent in all; on the 4th day they were still more marked, and there were scattered areas of necrosis, some of which were hæmorrhagic and others vesiculo-pustular. One of the lesions, which was excised and sectioned at this stage, showed numerous acidophilic intranuclear inclusions in the epithelial cells, which in addition to other changes will be described in detail separately. The remaining skin lesions became progressively worse, and did not begin to heal until about two weeks after injection; they were finally covered by large hæmorrhagic scabs, and scars were left at the sites. The monkey, however, did not appear ill, and daily temperatures revealed no fever. Thirteen days after the first injection it was re-inoculated intracutaneously (1·0 c.c.) and intraperitoneally (10·0 c.c.), and no appreciable skin or systemic reactions followed. Antiviral bodies against the B Virus were absent in the sera of both monkeys prior to the inoculations, but were present subsequently.

Although the failure to react upon re-inoculation and the development of antiviral bodies suggested that the skin lesions were probably specific, the finding of acidophilic, intranuclear inclusions of the type observed in rabbits injected with this virus indicated more definitely that the skin lesions in these monkeys were caused by the B Virus. It is therefore interesting to observe that the property which is most striking in rabbits, namely, the ready invasion of the central nervous system from peripheral foci, is not exhibited by the B Virus in rhesus monkeys, at least as far as can be ascertained clinically. As will be seen later in other monkeys, the intraperitoneal injection of B Virus gives rise to specific pathological changes in the peritoneum, omentum, and some of the viscera. That similar changes occurred in Monkey 3 without leading either to obvious invasion of the central nervous system or to a fatal issue is evident from certain post-mortem findings on this monkey when it died several months later following repeated bleedings; the liver and spleen were found to be covered with fibrous tissue, and almost completely adherent to the diaphragm, while the omentum was thickened and joined to the parietal peritoneum by many fibrous tissue bands. It may be noted that no such changes were found in Monkey 2 (killed several months later), which received its first intraperitoneal injection of virus at a time when it was already partly immune. The histopathology of these and other lesions referred to subsequently is being investigated in detail in collaboration with Dr. E. Weston Hurst, and will be reported at a later date.

SERIAL PASSAGE OF B VIRUS IN MONKEYS; EFFECT OF INTRACEREBRAL AND INTRAPERITONEAL INOCULATION.

When it was observed that in monkeys the B Virus could give rise to specific skin lesions without invading the central nervous system or leading to a fatal issue, it became desirable to determine whether a fatal disease, transmissible in series, could be established by some other route. This end was attained by means of intracerebral and intraperitoneal inoculations, and the results of serial passage are shown in Table I. The clinical pictures which followed these inoculations varied considerably, and may perhaps be conveyed best by abstracts of the protocols of the inoculated monkeys.

Protocols.

Macacus rhesus 4.

Previous history.—Adult monkey used for various experiments at the Institute for several years. On 16.i.34 inoculated intracerebrally with large dose of poliomyelitis virus, but remained well.

22.ii. Temperature before inoculation, 103·2° F.

Dose and route of inoculation: 1·0 c.c. intracerebrally, 10·0 c.c. intraperitoneally.

23.ii, 104·0°. Appeared normal in the morning.

6 p.m., appears ill; sits quietly in corner of cage; thighs flexed on abdomen, back bent anteriorly with head flexed on chest. No paralysis, paresis or tremors. Not excitable.

24.ii, 99·0°. In the morning no change in condition from that observed the previous evening. In the afternoon, however, it was found prostrate on the floor of the cage, with marked paresis of all the extremities. No convulsions noted.

25.ii, a.m., dead.

Post-mortem findings.—No gross changes discernible in brain, cord or any of the thoracic or abdominal viscera with the exception of the omentum, which appeared inflamed and was adherent

to the parietal peritoneum in the pelvis. Aërobic cultures of the heart's blood and brain were sterile. Rabbit 48 was injected intracutaneously with 0·4 c.c. of a suspension of the monkey's brain and cord; typical necrotic skin lesions developed at the inoculated sites, complete paralysis of the posterior extremities was present on the 7th day, and the rabbit was dead on the 8th day.

Macacus rhesus 5.

Previous history.—Small monkey used for poliomyelitis virus "neutralization" test about 5 weeks ago and remained entirely well.

14.iii.34. Temperature before inoculation, 101·8° F.

Inoculum: Centrifuged 20 p.c. suspension of brain and cord of Monkey 4.

Dose and route of inoculation: 1·0 c.c. intracerebrally on right side; 0·25 c.c. intracutaneously in each of two sites on right abdomen.

15.iii, 103·0°. Appears normal; skin practically negative.

16.iii, 104·0°. Appears normal; œdema and erythema about 2·5 × 1·5 cm. at both skin sites.

17.iii, 104·4°. Appears normal; central necrosis of both skin lesions.

18.iii, 103·5°. Appears normal; skin lesions about 3 × 3 cm. with central hæmorrhagic necrosis.

19.iii, 103·8°. Clumsiness of left upper and lower extremities.

20.iii, 107·0°. Partial spastic paralysis of left upper and lower extremities.

TABLE I.—*Serial Passage of B Virus in* Macacus rhesus *Monkeys.*

16th Passage Rabbit B Virus (Brain and Cord Suspension).

Monkey 4.
1 c.c. i.cereb., 10 c.c. i.p.
Signs 2nd day.
D. 8.

Monkey 5.
1 c.c. i.cereb., 0·25 c.c. i.cut. in 2 places.
Necrotic skin lesions.
Neurological signs 5th day. Recovered.

Monkey 6.
1 c.c. i.cereb., 6 c.c. i.p., 0·2 c.c. i.cut. 2 places.
Skin lesions.
Neurological signs 4th day.
D. 5.

Rabbit 48.
0·4 c.c. brain and cord suspension i.cut.
S_2 Par. 7, D. 8.

Monkey 7.
1 c.c. i.cereb., 0·2 c.c. i.cut.
Neurological signs 2nd day.
Prostrate 2nd day.
Sacrificed 2nd day.

Rabbit 98.
0·4 c.c. brain and cord suspension i.cut.
S_1 Par. 6, D. 7.

Monkey 8.
Berkfeld "V" filtrate. 8 c.c. i.v., 0·2 c.c. i.cut. 4 places.
Necrotic skin lesions.
Systemic disease. (Exanthem and enanthem—see text.)
Sacrified 15th day.

Monkey 9.
1·5 c.c. i.cereb., 10 c.c. i.p., 0·2 c.c. i.cut. 2 places.
Skin lesions.
Neurological signs 3rd day.
D. 4

Monkey 15.
1 c.c. i.cereb., 6 c.c. i.p.
Neurological signs 6th day.
Sacrificed 8th day.

Rabbit 108.
Berkfeld "V" filtrate. 0·2 c.c. i.cut.
S_1, Par. 8, D. 9.

Rabbit a.
0·4 c.c. brain suspension i.cut.
S_1, Par. 9, D. 11.

Rabbit b.
0·4 c.c. liver suspension i.cut.
S_1, Par. 7, D. 9.

Rabbit c.
0·4 c.c. spleen suspension i.cut.
S_1, Par. 6, D. 8.

Monkey brain and cord suspensions were used for passage. i.cereb. = intracerebrally; i.p. = intraperitoneally; i.v. = intravenously; i.cut. = intracutaneously. S_2, Par. 7, D. 8 = Skin lesion 2nd day, paralysis 7th day, dead 8th day after inoculation.

21.iii, 102·8°. Paralysis same; skin lesions more marked.
22.iii, 103·2°. Paralysis still more evident; questionable paresis of right lower extremity.
23.iii, not taken. General condition same; skin lesions more marked with more extensive necrosis.
27.iii, 103·0°. Papular eruption on inner aspect of right thigh, which has been rubbing against necrotic skin lesions on abdomen.
25.iii, 102·8°. Condition unchanged.
26.iii, 102·6°. Condition unchanged.
27.iii, 102·6°. Condition unchanged; hæmorrhagic scabs on skin lesions.
28.iii, 102·8°. Reinoculated with a suspension similar to that used on 14.iii.34.
Dose and route of inoculation: 1·0 c.c. intracerebrally on left side; 0·2 c.c. intracutaneously.
6·0 c.c. of uncentrifuged suspension intraperitoneally.

Monkey 6 was inoculated in the identical way on the same day, and it is interesting to compare the difference in the course.

29.iii, 103·4°. Site of new skin inoculation negative.
30.iii, 103·8°. Skin negative; generalized shivering or "tremors".
31.iii, 102·8°. Same.
1.iv, 103·0°. Old skin lesions healing with scar formation; new skin sites negative. Appears generally improved.
2.iv, 102·4°. Former paralysis becoming less marked.
3.iv, 102·4°. Former paralysis disappearing; skin negative.

In the course of the following two weeks the paralysis disappeared completely and no skin lesion developed at the site of the second inoculation. This monkey was sacrificed about four months later (30.vii.34), and a well-organized scar was found in the right cerebral cortex at the site of the first inoculation, whereas the left side appeared normal. This scar was undoubtedly the remains of an acute process some months before, and in view of the fact that the unilateral paralysis of the opposite side did not appear for five days after inoculation, it is quite likely that the acute process was of virus ætiology. That the B Virus is capable of causing such an acute localized process was observed in the case of Monkey 15, to be described later.

Macacus rhesus 6.

Previous history.—Had been used for poliomyelitis virus "neutralization" test about 5 weeks before and remained entirely well.

28.iii.34. Temperature before inoculation, 101·1° F.
Inoculum: Same as Monkey 5 on 28.iii.
Dose and route of inoculation: 0·2 c.c. intracutaneously in each of two sites; otherwise the same as Monkey 5 on same date.
29.iii. Temperature not taken. Appears entirely well; skin negative.
30.iii, 102·6°. Appears ill; small pink papules at both skin sites.
31.iii, 102·7°. Both skin lesions approximately 1 cm. in diameter with central red zones; occasional tremors.
1.iv, 102·8°. Skin same. Marked apathy and paresis; sits in corner of cage with thighs and thorax flexed on abdomen; cannot be made to stand up, and falls readily.
2.iv, 96·2°. Spastic paralysis of all extremities; head retracted; prostrate with gasping respirations. Dead in afternoon.

Post-mortem findings.—The significant gross pathological changes were observed in the abdomen. There was a diffuse fibrinous peritonitis; no free fluid; omentum inflamed and adherent to intestines and to parietal peritoneum in pelvis. Intestines adherent to each other. Organs were covered with fibrin and adherent to parietal peritoneum; few distinct yellowish, possibly necrotic zones seen on adrenals, but otherwise no gross changes were made out in any of the organs. Mesenteric lymph-nodes appeared enlarged. Aërobic cultures of heart's blood and brain were sterile. Rabbit 98 was injected intracutaneously with 0·4 c.c. of a brain and cord suspension of Monkey 6. Typical skin lesions with marked hæmorrhagic necrosis developed; the rabbit was paralysed on the 6th day, and dead on the 7th day.

Macacus rhesus 7.

Previous history.—Used for poliomyelitis virus "neutralization" test and remained entirely well.

5.iv.34. Temperature before inoculation, 103·7°.

Inoculum: 20 p.c. suspension in saline of brain and cord of Monkey 6 centrifuged at approximately 3000 r.p.m. for 15 minutes.

Dose and route of inoculation: 1·0 c.c. intracerebrally; 0·2 c.c. intracutaneously in each of two sites on abdomen.

6.iv, 103·0°. Appears entirely well; skin negative.

7.iv, a.m., 101·2°. Skin negative. Lethargy associated with marked tremors and spasticity of all extremities. Sits up and sleeps; readily roused, and when eyes are opened, nystagmus is observed.

P.m., 94·0°. Prostrate with spastic paralysis of all extremities; appears to be asleep most of the time; marked nystagmus in all directions observed when eyes are opened.

Sacrificed.

Post-mortem findings.—The only gross pathologic changes were observed in the meninges, which were markedly congested, and showed multiple petechial hæmorrhages on the inoculated side. Sections through the inoculated zone revealed no macroscopic reaction. The spinal cord showed no œdema but the meninges were congested. There was no peritonitis and the viscera showed no macroscopic changes. Aërobic cultures of the heart's blood and brain were sterile. Rabbit 108 was injected intracutaneously with 0·2 c.c. of a Berkfeld "V" filtrate of a suspension of the brain and cord of this monkey, and a typical necrotic skin lesion developed. There was evidence of biting and licking of the lesion on the 6th day, followed by complete paralysis on the 8th day and death on the 9th day.

Macacus rhesus 9.

Previous history.—Two weeks before inoculation with B Virus this monkey was inoculated with a mixture of potent neurotropic, H.F. and Perdrau EL_1 rabbit strains of herpes simplex as a control to the B Virus reactions, and to determine whether herpes administered in the same way as the B Virus would elicit any reactions in the monkeys used in this work. Herpes virus was injected intracutaneously in the form of rabbit brain suspension and testicular suspension (the latter gave typical herpetic skin reactions in rabbits), but neither gave the slightest skin lesion in the monkey. Herpes virus was also injected intracerebrally and intraperitoneally, but the monkey showed no fever and remained entirely well; at the end of fourteen days its serum, which before inoculation contained no herpeticidal antibodies, developed definite antiviral bodies for herpes.

16.v. Temperature before inoculation, 102·4° F.

Inoculum: Centrifuged 10 p.c. suspension in saline of brain and cord of monkey 7.

Dose and route of inoculation: 1·5 c.c. intracerebrally; 10·0 c.c. intraperitoneally; 0·2 c.c. intracutaneously in each of two sites.

17.v, 102·8°. Appears well; erythematous papules at both skin sites.

18.v, 103·2°. Appears well; œdema and erythema about 1 cm. in diameter at both skin sites.

19.v, 98·0°. Looks very ill. Nystagmus and generalized tremors. Sits up holding on to cage, thighs flexed on abdomen, back bent anteriorly with head flexed on chest. Closes his eyes frequently as if going off to sleep. Several convulsions were observed during the day; they usually began with a cry, and then the monkey would fall prostrate in a generalized convulsion which lasted about a minute.

20.v, less than 95·6°. Cold and prostrate with spastic paralysis of all extremities. Tends to sleep most of the time. Marked tremors when stimulated. Chewed an apple held close to his mouth, but could not swallow, and attempts to do so seemed to have set off a Jacksonian epileptiform convulsion. It began in the face with the eyes turning up and out, was followed by contraction of the right upper and lower extremities, and after a shriek, tonic and clonic convulsions of the entire body ensued. The entire episode lasted about one to two minutes. The monkey died early in the evening, and a post-mortem examination was performed soon after death.

Post-mortem findings.—The most significant changes were observed in the abdomen, where there was a marked fibrinous peritonitis with strands of fibrin extending between viscera and parietal peritoneum. The omentum appeared inflamed, and the lymph-nodes within it and the mesentery were enlarged. The intestines were adherent. Except that they were covered by fibrin the liver and spleen showed no change. No macroscopic changes were observed in the brain and cord or in any of the other viscera.

Macacus rhesus 15.

Previous history.—An apparently normal, healthy monkey recently admitted to the Institute and not used for any other investigation.

23.vi.34. Temperature before inoculation, 101·4° F.

Inoculum : 20 p.c. suspension of brain and cord of Monkey 7 kept in 50 p.c. glycerol for about 10 weeks.

Dose and route of inoculation : 1·0 c.c. of centrifuged supernatant liquid intracerebrally on right side ; 6·0 c.c. of uncentrifuged suspension intraperitoneally.

24.vi, entirely well ; 25.vi, 103·8°, appears ill, but no localizing signs ; 26.iv, 101·4°, same. 27.vi, 100·6°, same. 28.vi, 102·6°, same. 29.vi, 102·2°, appears very ill ; sits in corner of cage with thighs and chest flexed on abdomen. Partial paralysis of left upper extremity.

30.vi, 101·2°. Appears worse ; generalized paresis with paralysis of left upper and lower extremities.

1.vii, 97·6°. Very weak ; unable to stand up. Sacrificed.

Post-mortem findings.—Brain : Marked congestion of meninges with petechial hæmorrhages on right (inoculated) side. Hæmorrhagic necrosis in fronto-parietal region of cortex at site of inoculation with marked softening of surrounding brain tissue. Smear and culture of material from necrotic zone revealed no bacteria. The onset of unilateral paralysis of the opposite side 6 days after inoculation, taken together with the absence of bacteria or blood-clot in the necrotic zone, indicates that the process was most likely caused by the B Virus.

Abdomen : Marked adhesive fibrinous peritonitis. Omentum was thickened, inflamed and adherent to the intestines and parietal peritoneum ; omental and mesenteric lymph-nodes were enlarged. The liver was covered with a thin layer of fibrin, and both the surface and cross section showed innumerable tubercle-like necrotic foci about 1 mm. in diameter. The spleen was enlarged to about twice normal size ; its surface was covered with a thin layer of fibrin, and numerous tubercle-like necrotic foci were present. The adrenals were also enlarged to about twice their normal size, with almost complete replacement of the parenchyma with necrotic foci. The kidneys and lungs appeared entirely normal.

Tests for virus.—Suspensions of brain (from necrotic zone), of liver and spleen were injected intracutaneously into individual rabbits. Typical necrotic skin lesions appeared, followed by biting of the skin at the level of inoculation after 5 to 8 days in the different rabbits, with ensuing paralysis and death.

Remarks on Above Protocols.

The results obtained with Monkeys 2 and 3 showed that intracutaneous and intraperitoneal injections of B Virus did not give rise to any perceptible neurological signs, and were not fatal. The neurological signs which followed intracerebral inoculation of the virus varied in different monkeys. While progressive paresis and spastic paralysis were shown by all, in two monkeys (5 and 15) the first signs were unilateral, pointing to a more marked pathological change at the site of inoculation, which was borne out by a macroscopic examination of their brains. In two other monkeys (7 and 9) marked nystagmus and somnolence were striking signs, and epileptiform convulsions were observed only in one (9). None showed any appreciable fever, while a subnormal temperature for 24 to 48 hours pointed to a fatal issue. The monkeys which received intraperitoneal inoculations of virus assumed a posture which suggested peritoneal irritation, and indeed all showed evidence of an acute fibrinous peritonitis. The fact that the virus suspensions used for inoculation were all bacteriologically sterile, and that the intraperitoneal injection of normal and herpetic brain suspensions does not give rise to a similar picture, suggests that the peritonitis encountered in these monkeys was probably caused by the B Virus. Macroscopic evidence of involvement of the abdominal viscera was present in one monkey only, and in this case suspensions of the liver and spleen were shown to contain the virus. Histological investigations

which are now in progress, and which will be reported in detail in a separate communication, revealed acidophilic, intranuclear inclusions in the parenchymal cells of some of the viscera, and in endothelial cells of the omentum, indicating that both the peritonitis and the visceral lesions are caused by the B Virus.

The virus has now been transmitted in series through four generations of *Macacus rhesus* monkeys, and suspensions of the brain and viscera of the last passage when injected intracutaneously into rabbits gave rise to the typical B Virus disease in them; furthermore, the finding of identical intranuclear, acidophilic inclusions in the skin, and visceral lesions of the monkey, together with the fact that convalescent monkey sera neutralize the rabbit B Virus, leave little doubt that both the rabbit and monkey diseases are caused by the same virus. It is interesting to note, however, that signs of involvement of the central nervous system from a peripheral focus, which are so striking a part of the picture in rabbits, are apparently entirely suppressed in the monkey. Whether or not absence of clinical signs is associated with complete absence of involvement of the central nervous system in these cases has yet to be determined. Still it should be pointed out that four serial passages in the monkey did not modify the virus in any way for the rabbit; whether or not a greater number of monkey passages could modify the virus by completely suppressing the "neurotropic" invasive property even for rabbits is problematical, and could be decided only by experiment.

EFFECT OF INTRAVENOUS INOCULATION OF B VIRUS.

The purpose of this experiment was to determine the course of the disease resulting from introduction of the virus directly into the venous circulation. A Berkfeld "V" filtrate of the brain and cord of Monkey 7 was used for this purpose. Some of the filtrate was also injected intracutaneously for a triple purpose: first, to determine whether or not the three brain-to-brain passages which the virus had now undergone in the monkey had modified the skin lesion; secondly, to observe the difference, if any, in a skin lesion produced with a Berkfeld filtrate from that produced with a crude suspension; and thirdly, as a check on the pathogenicity of the filtrate for the monkey. The course of events may be seen best in the protocol of the inoculated monkey:

Macacus rhesus 8.

Previous history.—This was a small monkey; it had been used for a poliomyelitis virus neutralization test on 13.ii.34, and remained entirely well.

16.iv. Temperature before inoculation, 103·2° F.

Inoculum: Berkfeld "V" filtrate of 15 p.c. suspension in saline of brain and cord of Monkey 7.

Dose and route of inoculation: 8·0 c.c. intravenously; 0·2 c.c. intracutaneously in each of four sites on right side of abdomen.

17.iv, 103·0°. Pink papules about 1·0 cm. in diameter at injected sites.

18.iv, 104·8°. Œdema and erythema 2·0 cm. in diameter at injected sites.

19.iv, 104·8°. Numerous scattered necrotic patches (about 2 mm. each) on skin lesions; two of the lesions excised for study.

20.iv, 105·0°. Remaining skin lesions are larger and necrotic in centre. On both legs, at the sites where venepuncture was unsuccessfully attempted and slight leakage probably occurred, there are now definite skin lesions. Lymphangitis radiating from these to the femoral lymph-nodes is discernible; the lymph-nodes are enlarged. A necrotic patch is present on the buccal mucous membrane and greyish-white spots are seen on the tongue.

21.iv, 104·8°. Tongue lesions are more marked, consisting of greyish-white, necrotic zones about 2 to 3 mm. in diameter. Similar lesions are now present at the corner of the mouth and on mucous membranes of the lips.

22.iv, 104·6°. Condition appears to be the same.

23.vii, 104·8°. Looks very ill and is quite weak. Face and eyelids are swollen. Some pock-like lesions about lips, nose and eyes. Enanthem more marked with lesions on palate. Skin lesions on legs are now vesiculo-pustular.

24.iv, 104·6°. Lesions on face and legs are more numerous; abdominal skin lesions show marked central necrosis. Shivering or tremors.

25.iv, 103·0°. Bilateral conjunctivitis. Lesions on the face are depressed in the centre and present a crater-like appearance. More lesions have developed apparently along the lymphatic spread on the legs, which seem to be very painful to the touch.

26.iv, a.m., 100·0°. Marked necrosis of all skin lesions; most of enanthem gone and the tongue appears very smooth. Gross tremors.

P.m., 101·5°.

27.iv, 99·0°. Tremors continue.

28.iv, 101·5°. Marked tremors. Œdema of face gone. Conjunctivitis practically disappeared. Coughs and sneezes. Not very active; sits huddled up in corner of cage shivering.

29.iv, 101·3°. Tremors continue. Bleeding ulcers in mouth. Skin lesions still badly ulcerated. Coughing.

30.iv, 103·0°. Appears generally somewhat improved. New ulcers on tongue, however, and coughing continues.

1.v, a.m., 103·8°. Condition same.

P.m., 101·7°. Bled and sacrificed.

Post-mortem findings.—Chest: Left lung adherent to diaphragm and to remainder of parietal pleura, with evidence of an organizing acute pleuritis. There is consolidation of the entire left lower lobe with a purulent necrotic centre; no gross evidence of tuberculosis (there was also no microscopic evidence of tuberculosis). Similar consolidation of right middle lobe. *Abdomen:* With the exception of the left adrenal, which shows suspicious necrosis, the other organs appear normal. The lymph-nodes appear rather larger than normal. *Brain and cord:* No gross evidence of pathological change.

The blood taken just before death, *i. e.* 15 days after inoculation, contained antiviral bodies for the B Virus. An attempt was made to inoculate another normal monkey intravenously, with filtered virus, but the monkey died suddenly, before even 3 c.c. were injected, the death apparently being due to the well known, but little understood, toxicity of homologous organ extracts. It may be suggested here that such death might be avoided by the administration of the total dose in small portions at intervals of 5 minutes. In another experiment it was shown that following intravenous inoculation, Monkey 5, which had been rendered immune to B Virus, developed none of the lesions exhibited by Monkey 8.

A review of the protocol of Monkey 8 shows that intravenous inoculation of the virus apparently gives rise to a clinical picture markedly different from that which follows either the intracerebral and the intraperitoneal, or the intracutaneous and intraperitoneal inoculations. The most striking feature undoubtedly is the appearance of the exanthem and enanthem. The exanthematous lesions were most striking about the forehead, the eyes and the face; the enanthem was particularly apparent on the buccal mucous membranes, the palate, the lips and the tongue, but a definite conjunctivitis was also present for about 3 days. There was a sustained fever for 7 days. The clinical picture presented by this monkey simulated most markedly that which follows the intravenous injection of large amounts of vaccinia in rhesus monkeys, as described by Camus (1917–18). The severity of the lesions at the

sites of intracutaneous inoculation indicates that the brain-to-brain passages which the virus had undergone had not modified its capacity for producing skin reactions. These skin lesions in Monkey 8 again bore a rather close macroscopic resemblance to those produced by vaccinia in the rhesus monkey ; as in the previous cases, however, these also showed acidophilic intranuclear inclusion bodies, and it has also been shown elsewhere (Sabin, 1934*a*) that there is no immunological relationship between the B Virus and vaccinia.

NATURAL B VIRUS INFECTION AMONG *MACACUS RHESUS* MONKEYS.

The difficulty encountered in America in transmitting the B Virus to *Macacus rhesus* monkeys suggests now the possibility that a large number of these were immune to the virus, probably as a result of natural infection. A review of the data on the attempted transmission of the virus, using the original human tissue, indicates that one of the inoculated monkeys probably succumbed to the virus. Gay and Holden (1933), in New York, who isolated what would appear to be the same virus from the human material, were also unable to transmit it to rhesus monkeys ; they state that of 7 " normal " rhesus sera, 4 neutralized the virus in rabbits, and that one of these sera was effective against this virus only. In view of the fact that no difficulty was encountered in the serial transmission of the B Virus in rhesus monkeys in London, it appeared desirable to test a group of " normal " rhesus sera for their capacity to neutralize the virus. Gay and Holden (1933) pointed out that the sera of a large percentage of rhesus monkeys contain herpeticidal antibodies ; the origin and nature of these " antibodies " is not clear, since this species of monkey has been shown to be insusceptible to herpes simplex. However, since it was demonstrated in a previous investigation (Sabin, 1934*a*) that certain very potent antiherpes sera may also neutralize minimal quantities of B Virus, all the monkey sera tested against B Virus in the present study were also titrated for herpes antiviral bodies. The technique used for the B Virus and herpes neutralization tests has already been described (Sabin, 1934*a*) ; simultaneous titrations of both viruses were performed in each case to permit of a more or less quantitative expression of the data, which are presented in Table II. As may be seen in the table, the tests were designed to detect even minimal quantities of antiviral substance for both viruses. Of 13 monkey sera, only 1 neutralized B Virus, while 5 neutralized herpes in greater or lesser degree. One of the monkeys (9), whose serum neutralized neither virus, developed herpeticidal antibodies following the inoculation of a large quantity of herpes virus by various routes, but none against B Virus. The one monkey (11) whose serum contained B Virus antiviral bodies was bled the day after its admission to the animal house, so that it apparently had those antibodies before coming to the Institute. Furthermore the titre of this serum against B Virus was as high as, and perhaps even higher than, that of the best hyperimmune serum artificially prepared thus far. Unfortunately this monkey was used for other work, and could not be tested for active immunity against the B Virus ; it succumbed to an infection with equine encephalomyelitis given by Dr. Hurst. It is highly probable, therefore, that some time before coming to the Institute this monkey

had a natural infection with B Virus, and it is not unlikely that B Virus infection may be enzootic for these monkeys in certain regions.

DISCUSSION.

The object of the present investigation was not only to learn more of the properties of the B Virus, but also, by determining whether or not rhesus monkeys might be affected by it under natural conditions, to establish a link between the monkey-bite and the ensuing human disease. The results of this study clearly indicate that the B Virus is pathogenic for *Macacus rhesus* monkeys. It was transmitted in series through four generations by intracerebral inoculation, and the final monkey material produced the same disease in rabbits as that with which passage was started. It was found to produce distinct lesions in the brain, skin, peritoneum, and some of the viscera. The ætiological relationship of the B Virus to these lesions was established by inoculation of rabbits with suspected material, and microscopically by the demonstration of acidophilic, intranuclear inclusion bodies. Intravenous inoculation of the virus gave rise to a generalized systemic disease, with an exanthem and enanthem closely resembling that which is produced by vaccinia given by the same route. The clinical and gross pathological picture of experimental B Virus disease in rhesus monkeys had also other features in common with that produced by vaccinia; the two are readily differentiated, however, by microscopic examination of the lesions and by the inoculation of rabbits. A striking feature of the B Virus in these monkeys is that it apparently does not give rise to central nervous system signs following peripheral inoculation, which is one of its most important and constant properties in rabbits.

The question whether monkeys may be infected with this virus in nature, or not, is important both with regard to determining its natural habitat, and as a link in establishing the origin of the human disease. That natural infection of monkeys with B Virus probably does occur is now supposed on the basis of the following observations. First, the majority of the rhesus monkeys which were available for this study in New York were immune, so that it proved impossible to establish the virus in them; secondly, among 13 "normal" healthy monkeys in London, 1 was found whose serum "neutralized" the B Virus as well as the best hyperimmune serum, while the sera of the other 12 completely failed to "neutralize". Because the B Virus tends to be localized, and does not invade the central nervous system in these monkeys, it is quite possible that the natural disease may be relatively mild in them. Yet if some of the virus were to find its way to another species, in which it is more invasive, a fatal result might readily ensue. The character of the human disease, as previously described (Sabin and Wright, 1934), indicates that in that case it functioned more as it does in the rabbit with regard to its invasiveness of the central nervous system; whether or not this neuro-invasive property of the virus obtains for all human beings, as is the case with rabbits, or only for some of them as in the case of guinea-pigs, is, of course, problematical. The skin lesions on the human fingers, at the sites of the monkey-bite, were much like those produced experimentally in the monkey, while the subsequent spread to the nervous system and involvement of the viscera is more characteristic of the action of the virus in rabbits. Still, it

TABLE II.—*B. virus and Herpes Simplex Antiviral Bodies in Sera of "Normal" Macacus rhesus Monkeys.*

Serum of monkey—	Against B virus.			Against herpes simplex.*		
	Dilution of virus.	M.I.S.D.†	Result.	Dilution of virus.	M.I.S.D.	Result.
No. 1	1 : 20	n.d.‡	S_3, Par. 7, D. 9§	1 : 20	5,< 50	Herpetic lesion.
	1 : 200	„	S_5, Par. 9, D. 10	1 : 100	1,< 10	„ „
	1 : 1000	„	S_4, Par. 9, D. 10			
„ 2	1 : 20	„	S_3, Par. 7, Sac. 8	1 : 20	5,< 50	No lesion.
	1 : 200	„	S_3, Par. 8, D. 9	1 : 100	1,< 10	„
	1 : 1000	„	S_7, Par. 11, D. 13			
„ 3	1 : 200	„	S_2, Par. 7, D. 8	1 : 20	100,< 500	Herpetic lesion.
	1 : 1000	„	S_4, Par. 9, D. 10	1 : 100	20,< 100	„ „
				1 : 20	50,< 500	„ „
				1 : 100	10,< 100	„ „
				1 : 1000	1,< 10	„ „
„ 4	1 : 200	„	S_2, Par. 8, D. 9	1 : 20	100,< 500	„ „
	1 : 1000	„	S_6, Par. 9, D. 11	1 : 100	20,< 100	„ „
No. 9 before inoculation with herpes simplex	1 : 500	2,< 20	S_2, Par. 8, D. 8	1 : 20	100,< 500	„ „
				1 : 100	20,< 100	„ „
				1 : 1000	2,< 10	„ „
No. 9 after inoculation with herpes simplex	1 : 500	2,< 20	S_3, Par. 8, D. 9	1 : 20	100,< 500	„ „
				1 : 100	20,< 100	No lesion.
				1 : 1000	2,< 10	„
No. 10	1 : 500	2,< 20	S_3, Par. 12, D. 14	1 : 20	100,< 500	Herpetic lesion.
				1 : 100	20,< 100	„ „
				1 : 1000	2,< 10	No lesion.
No. 11 undiluted	1 : 500	2,< 20	Negative	1 : 20	100,< 500	„
	1 : 100	10,< 100	„	1 : 100	20,< 100	„
	1 : 20	50,< 500	„	1 : 1000	2,< 10	„
No. 11 diluted 1 : 10	1 : 20	500	„	1 : 20	100,< 500	Herpetic lesion.
„ „ 1 : 50	1 : 20	500	„	1 : 20	100,< 500	„ „
„ „ 1 : 100				1 : 20	100,< 500	„ „
„ „ 1 : 500						

No. 12	1 : 500	2,<20	S_4, Par. 9, D. 10	1 : 20	100,<500	Herpetic lesion.		
				1 : 100	20,<100	„	„	
				1 : 1000	2,<10	„	„	
„ 13	1 : 500	2,<20	S_3, Par. 9, D. 12	1 : 20	100,<500	Doubtful lesion.		
				1 : 100	20,<100	No lesion.		
				1 : 1000	2,<10	„		
„ 14	1 : 500	2,<20	S_3, Par. 8, D. 10	1 : 20	50,<500	Herpetic lesion.		
				1 : 100	10,<100	„	„	
				1 : 1000	1,<10	„	„	
„ 15	1 : 500	2,<20	S_4, Par. 8, D. 9	1 : 20	50,<500	„	„	
				1 : 100	10,<100	„	„	
				1 : 1000	1,<10	„	„	
„ 16	1 : 500	2,<20	S_4, Par. 8, D. 9	1 : 20	100,<500	„	„	
				1 : 100	20,<100	„	„	
				1 : 1000	2,<10	„	„	
„ 17	1 : 500	2,<20	S_2, Par. 9, D. 10	1 : 20	100,<500	No lesion.		
				1 : 100	20,<100	„		
				1 : 1000	2,<10	„		

* The herpes simplex virus used in these tests was the Perdrau EL_1 strain in the form of centrifuged 10 p.c. suspensions of rabbits' testes removed 48 to 72 hours after inoculation with glycerinated herpetic brain.

† M.I.S.D. = Minimal infective skin dose; for B virus this represented the minimal amount required to produce paralysis and death; for herpes simplex, the minimal amount required to produce a characteristic skin lesion.

‡ n.d. = Not determined.

§ S_3, Par. 7, D. 9 = skin lesion first appeared on the 3rd day, paralysis on the 7th day, and death on the 9th day.
All tests carried out on rabbits.

may readily be seen how the bite of a monkey infected with, or carrying, the B Virus could produce the entire disease. There is thus another instance of a virus which, though relatively harmless in one host under natural conditions, may yet give rise to a fatal disease when transmitted to another.

SUMMARY.

1. The B Virus is pathogenic for *Macacus rhesus* monkeys, and has been transmitted through four generations by intracerebral inoculation.

2. Intracerebral inoculation of the virus is usually fatal, giving rise to varying clinical syndromes.

3. The most striking features of the disease following the intravenous inoculation of the virus are an exanthem (chiefly about the forehead, eyes and face) and enanthem (buccal mucous membranes, tongue, palate, conjunctivæ), which closely resemble the picture produced by the intravenous inoculation of vaccinia in rhesus monkeys.

4. Intracutaneous injection of the virus is followed by acute inflammation with necrosis, which may be hæmorrhagic or vesiculo-pustular. Macroscopically the lesion bears some resemblance to that produced by vaccinia, but microscopically it is readily differentiated from the latter by the presence of acidophilic intranuclear inclusions in the epithelial cells, and by the absence of Guarnieri bodies.

5. Intraperitoneal injection of the virus gives rise to an acute peritonitis, with occasional invasion of some of the abdominal viscera.

6. In monkeys peripheral inoculation of the virus does not lead to any apparent clinical involvement of the central nervous system, and in this respect the virus lacks one of its most striking and constant properties in rabbits.

7. Among 13 " normal " monkey sera, only one was found to contain antiviral bodies for the B Virus, and the titre of this particular one was as high as that of the best hyperimmune serum ; on this basis it is believed that the monkey may at one time have had a natural B Virus infection.

8. Similarly, the opinion is expressed that the reason for the failure to establish this virus in rhesus monkeys in New York is that the majority of them were immune, probably as a result of natural infection, and that the human disease from which the B Virus was isolated was caused by the bite of a monkey which was infected with it.

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ADDENDUM.

Since this paper was submitted for publication, the sera of two additional, normal rhesus monkeys were found to contain antiviral bodies for the B Virus. These monkeys were also shown to possess an active immunity as evidenced by their failure to give any appreciable skin reactions following the intracutaneous injection of the virus.

OBSERVATIONS CONCERNING THE MECHANISM OF PARATHYROID HORMONE ACTION.

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A SURVEY of the available data has appeared to substantiate the view that the parathyroid hormone exerts its effect on calcium metabolism by a direct action on the bones (Thomson and Collip, 1932). Selye (1932*a*), and Pugsley and Selye (1933), have shown that the liberation of calcium salts from the skeleton in the acute stage of experimental hyperparathyroidism occurs simultaneously with the appearance of large numbers of osteoclasts, while in the chronic stage calcium salts are deposited in the bones by means of osteoblastic bone apposition. They considered the changes in the calcium metabolism to be secondary to these changes in the osseous system. Ellsworth (1932), however, observed that the injection of parathyroid extracts in human subjects led immediately to an increased urinary excretion of phosphate ; he attributed this to a lowering of the renal threshold, and suggested that it might be the cause of the subsequent rise in the level of the serum calcium. We have felt unable to accept this suggestion, since Thomson and Pugsley (1932) produced hypercalcæmia in dogs, with no concomitant fall in plasma phosphate, and we wish now to report further and decisive evidence against it.

We carried out complete removal of the kidneys in eight male albino rats of Wistar stock, weighing 60 to 80 g. each. The animals were injected with 40 units of parathyroid hormone twice daily for two days, and killed 48 hours after the operation. The lower ends of the femurs were decalcified with nitric acid, stained with hæmatoxylin and eosin and examined histologically : all showed marked signs of osteoclastic bone resorption. We conclude that the action of the parathyroid hormone on the bones is independent of any direct influence it may have on the renal threshold for phosphates.

Taylor, Weld, Branion and Kay (1931) have advanced the theory that very large doses of irradiated ergosterol stimulate the production of the parathyroid hormone, and thus produce decalcification of the bones. Selye (1932*a*), however, pointed out that the decalcification produced by excessive dosage with irradiated ergosterol is histologically different from that observed in hyperparathyroidism, and is not accompanied by the appearance of large numbers of osteoclasts. This latter point has recently been confirmed by Ham and Lewis (1934), who feel nevertheless that their " results are compatible

with, although they do not directly support, the theory that vitamin D acts through the intermediary of the parathyroid mechanism to control a fraction of the serum calcium ". We regard it as highly improbable that irradiated ergosterol should produce changes in the bones entirely different from those observed in hyperparathyroidism, if its action were merely to stimulate the hormone production of the parathyroid glands ; nor do we feel that the fact that decalcification occurs without participation of osteoclasts in hypervitaminosis D refutes the suggestion that the osteoclasts observed in hyperparathyroidism play an active part in the decalcification typical of that condition.

Ham and Lewis (1934) found that osteoid tissue is formed in the bones of rats treated with large amounts of irradiated ergosterol, and consider this condition to be a special type of rickets. We have described the histogenesis of this osteoid tissue in detail (Selye, 1932*a*), and interpreted it as a secondary reaction to the death of bone, which is usually the result of chronic ergosterol overdosage ; the condition is, in our opinion, quite distinct from the characteristic lesions of rickets. We may take this opportunity of pointing out that whereas Shelling, Asher and Jackson (1933) ascribe to us a claim that parathyroid overdosage produces true rickets, we have stated in discussing this condition (Selye, 1932*b*) that " although these changes are suggestive of rickets produced by dietary means, no conclusion as to whether or not the two lesions can be considered identical is possible ".

SUMMARY.

The characteristic effect of the parathyroid hormone on the bones of rats is obtainable after bilateral nephrectomy, and hence cannot be ascribed to an action of the hormone on the renal threshold for phosphate. Since the histological changes in the bones in hypervitaminosis D are entirely different from those observed in experimental hyperparathyroidism, it is difficult to believe that irradiated ergosterol acts merely by stimulating production of the parathyroid hormone.

The parathormone used in these experiments was kindly supplied by the Eli Lilly Company.

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IMMUNIZATION OF ANIMALS WITH FORMOLIZED TISSUE CULTURES OF *RICKETTSIA* FROM EUROPEAN AND MEDITERRANEAN TYPHUS.

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UNTIL recent years it was considered impossible to immunize animals with dead typhus virus. Phenolized or heated emulsions of infected brain or spleen tissue failed to confer immunity. Blanc (1916) gave one injection of a heavy suspension of triturated brain from infected guinea-pigs, heated to 55° C. for 30 minutes, with negative results. Da Rocha-Lima (1917, 1918) used phenolized brain, spleen, and kidney suspensions, and gave three successive injections of 2·5, 2·5 and 3·0 c.c. ; when tested for immunity, 3 out of 5 guinea-pigs developed a typical infection. Doerr and Pick (1918) also used phenolized brain emulsions ; 8 guinea-pigs were given three subcutaneous injections of 0·05 to 0·1 g. of brain tissue ; none of the animals proved immune. Landsteiner and Hausmann (1918) employed brain emulsions kept 14 days at 0° C., or 7 days frozen ; but, although in some instances there was a modification of the temperature, no definite immunity was noted. Doerr and Schnabel (1919), in a critical study of the various methods of immunization recorded above, concluded that none of them conferred immunity on guinea-pigs.

In an extensive series of experiments, Kligler and Olitzki (1932) found that 0·1 to 0·3 g. of infected brain tissue in which the virus had been killed failed to induce immunity in guinea-pigs. When large quantities of brain (3 g.) were injected, some of the guinea-pigs treated developed a partial immunity manifested by a modified temperature. Complete immunity against the same infecting dose could only be produced with small doses of infected brain tissue treated for one or two hours with 0·1 p.c. formalin, or with distilled water. This material still contained live virus in small amount or attenuated in virulence, and the resulting immunity was due to an inapparent infection.*

This brief summary of the literature indicates clearly that killed tissue virus fails to produce immunity, except when large amounts of tissue are used, and even then the immunity is only relatively slight.

More satisfactory results have been obtained with phenolized or formolized louse virus. Da Rocha Lima (1918) was the first to report positive results by injecting phenolized material from 35 infected lice per guinea-pig. Doerr and Schnabel (1919) repeated these experiments with negative results, although they used larger numbers of lice. Subsequently, however, Breinl (1924), and Kligler, Olitzki and Aschner (1931) confirmed and extended the findings of Da Rocha Lima. These authors used lice infected by the Weigel technique. Breinl gave one injection of phenolized virus, using the material from 20 lice

* Unpublished experiments.

for each guinea-pig, while Kligler, Olitzki and Aschner gave 5 successive injections of formolized virus, the total amount per guinea-pig being equivalent to the material from 12 lice.

A further advance toward the solution of this problem was made by Zinsser and Castaneda (1931), who demonstrated the immunizing property of formolized suspensions of *Rickettsia* from the tunica vaginalis of guinea-pigs, or from the peritoneal washings of rats, infected with the Mexican type of typhus.

The failure of the Mexican virus to immunize guinea-pigs against the virus of European or Epidemic typhus (Zinsser and Castaneda, 1931), and the difficulty of maintaining large colonies of lice for this purpose, led us to seek another solution to this problem. The experiments reported below pertain to the use of killed tissue cultures of *Rickettsia* as an immunizing vaccine.

PREPARATION OF CULTURES.

(a) *Virus of European or Epidemic Typhus.*

The cultivation of pathogenic *Rickettsia* is possible only in the presence of live tissue (Wolbach and Schlessinger, 1923; Zinsser and Batcheldor, 1930; Pinkerton and Hass, 1931). Nigg and Landsteiner (1932) have succeeded in cultivating the *Rickettsia* of Mexican typhus in flask cultures by the procedure used by Rivers for vaccine virus, using infected tunica vaginalis as a source of *Rickettsia* and normal tunica as tissue.

Our attempts to employ this technique for the cultivation of *Rickettsia* from a strain of European typhus failed until we used *Rickettsia* from infected lice to initiate the cultures (Kligler and Aschner, 1933). Tunica from guinea-pigs infected with European typhus are practically free of *Rickettsia*, consequently successful cultures and serial subcultures of the European virus can best be achieved if infected lice guts are used to initiate the culture.

The procedure is as follows: Infected lice, 8–10 days after infection, are treated with alcohol and washed with sterile water. The gut is then removed aseptically and placed in a drop of saline in a sterile hollow slide. The slide is put in a sterile Petri-dish, which is kept on ice to prevent drying. The tunica of a guinea-pig is then macerated together with the lice guts, and the material divided among five or six small Erlenmeyer flasks containing 3·0 c.c. of medium. The medium consists of 5 parts Tyrode solution and 1 part fresh guinea-pig serum. The flasks are stoppered with sterile rubber stoppers and incubated at 28°–30° C. The medium is slightly alkaline (pH 7·2), and practically remains so, or approaches neutrality, as long as the bottle is properly stoppered. After 8–10 days a rich culture is obtained, many of the cells being packed with *Rickettsia*. Subcultures are made in the same manner as above, using the infected tissue in place of the louse guts.

(b) *Virus of Mediterranean or Rat Typhus.**

Since this virus produces scrotal lesions, cultures can be initiated by using the infected tunica. We have found that the simplest procedure is to macerate the infected tunica and divide the material among 5 or 6 flasks; normal

* We are grateful to Dr. Lepine for sending us white rats infected with a strain of this virus.

tunica is not required. In 7 to 8 days massive cultures are obtained, most of the cells being infected. The cultures can be made 4 to 7 days after the animal has been infected, and thus far this method has given 100 p.c. positive cultures.

PREPARATION OF THE VACCINE.

Old as well as young cultures can be used for preparing the vaccine. As a rule we use cultures 2 to 3 weeks old. Large centrifuge tubes fitted with glass pestles, both ground to fit, serve as mortars for grinding up the infected tissue. The tubes and pestles are sterilized separately. The culture material is removed from the flasks to these centrifuge tubes, care being taken to transfer all the tissue. If necessary the flasks are washed with a small amount of saline, and the washings added to the centrifuge tubes. The material is then centrifugalized at high speed (2500–3000 revolutions) for one-half to three-quarters of an hour. The supernatant fluid is then removed, and the sediments thoroughly triturated. To assure the complete break-up of the cells, the sediment is frozen and thawed twice and the material again triturated. The emulsion is then diluted with the original supernatant fluid, or with an amount of saline equal to the culture fluid removed. Formalin is then added to give a final concentration of 0·1 p.c. This vaccine is sterile and non-infective even in large doses. In fact the organisms in the cell-free suspension are highly sensitive, and apparently die even if kept only 24 hours at 37° C. All the immunization experiments reported below have been made with stock vaccines prepared in this manner.

IMMUNIZATION OF GUINEA-PIGS WITH EUROPEAN VIRUS.

Experiment 1.—Two guinea-pigs received three intraperitoneal injections of formolized vaccine, 1·0 c.c., 0·75 c.c. and 1·0 c.c. respectively. Each animal thus received material equivalent to about one culture, or one-sixth of a tunica. The injections were given on 24.xi, 27.xi and 1.xii respectively. During the immunization the temperature remained entirely normal. Ten days after the last injection the animals were given 80 infective doses of brain virus and temperatures taken daily for two weeks. The temperatures of the treated animals remained normal, while those of the controls inoculated at the same time ran a typical course. At this time the guinea-pigs received ten times the previous dose of brain virus without any reaction. Typical temperature curves of immunized animals and corresponding controls are shown in the accompanying chart (Chart 1).

Experiment 2.—Four guinea-pigs were given three successive injections of formolized vaccine prepared from a mixture of stock cultures. The cultures were 4th and 5th subcultures, and were from 14 to 27 days old. The vaccine was tested both for sterility and virulence. One c.c. of vaccine was injected intraperitoneally on 30.i, on 2.ii and on 5.ii. On 11.ii the animals were given 80, and on 22.ii, 800 infective doses of brain virus. None of the immunized animals showed any reaction, while the corresponding controls developed typical infections (see Chart 1).

It should be noted that the cultures are very sensitive. Mere freezing and thawing, even without the addition of formalin, renders them non-infective.

This observation has been made repeatedly. Not only is the culture non-infective, but even massive doses fail to produce an apparent infection. The following instance is illustrative. A guinea-pig was given 1 c.c. of the mixed cultures used for the preparation of the vaccine for the above experiment. The animal remained normal. Three weeks later (22.ii) this guinea-pig was infected along with those immunized, and developed a typical infection.

Experiment 3.—The previous experiment was repeated using 6th and 7th generation subcultures. Four guinea-pigs received three injections each, as in the previous experiment. On reinfection they all proved immune. The dose used (3 injections of 1·0 c.c.) produced no rise in temperature during the period of immunization, and rendered the animals immune against subsequent infection.

IMMUNIZATION OF ANIMALS WITH CULTURES OF THE RAT VIRUS.

A similar series of experiments were made with cultures of the Mediterranean or rat virus. The cultures were made directly from the infected tunica, and the vaccines were prepared in the manner described above. The immunity of the animals was tested with the homologous tissue virus.

Immunization of Guinea-Pigs.

Experiment 1.—Six guinea-pigs were immunized, two receiving 3 injections of 1·0 c.c., and four 0·5 c.c. of the vaccine. The injections were given 29.iv, 2.v and 7.v respectively. None of the animals showed any temperature reaction during vaccination. On 17.v, 10 days after the last injection, the animals received 0·5 c.c. of an infected tunica suspended in 20 c.c. of saline. The controls developed typical infections, while the treated animals remained normal.

Experiment 2.—This is essentially a repetition of the previous experiment, with the exception that one set of 3 animals received three 1·0 c.c. injections of saline vaccine alone, and another set received in addition three injections of 1·0 c.c. of the formalized supernatant fluid of the cultures. All the animals developed solid immunity to the infective dose used (0·5 c.c. of an infected tunica suspended in 20 c.c. saline), and no difference in the degree of immunity was noted.

Experiment 3.—This experiment was designed to ascertain the minimal dose of vaccine required for immunization as well as the immunizing value of the clear supernatant fluid of the culture. Five sets of guinea-pigs were used:

A. Two guinea-pigs received three 1 c.c. injections (i.p.) of supernatant fluid.

B. Two guinea-pigs received three 2 c.c. injections (i.p.) of supernatant fluid.

C. Five guinea-pigs received three injections (i.p.) of 0·25 c.c. of the formalized saline suspension of the culture.

D. Five guinea-pigs received three injections (i.p.) of 0·5 c.c. of the same suspension.

E. Five guinea-pigs received three injections (i.p.) of 1·0 c.c. of the same suspension.

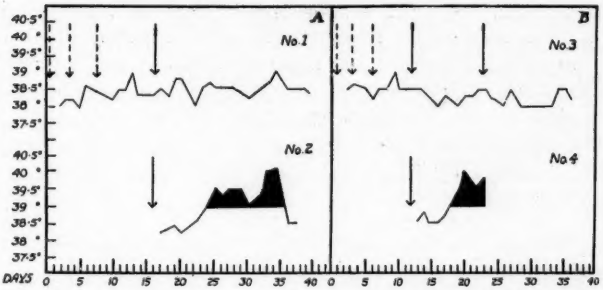

CHART 1.—Typical reactions of guinea-pigs immunized with formolized culture-vaccine of European strain. A = Experiment 1: (1) Immunized guinea-pig; (2) control. B = Experiment 2: (3) Immunized guinea-pig; (4) control. Dotted arrows = days when vaccine injected. Full arrow = day of infection.

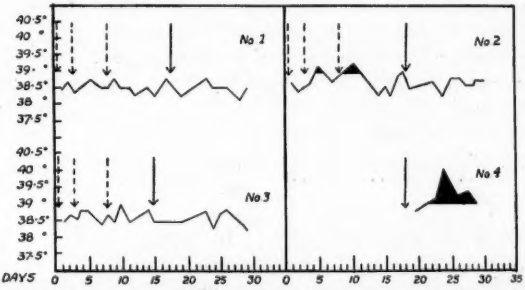

CHART 2A.—Temperature curves of guinea-pigs immunized with formolized *Rickettsia* cultures of the rat strain. Nos. 1 and 2 immunized guinea-pigs infected with rat strain. No. 3 immunized guinea-pigs infected with European strain. No. 4 Control. Dotted arrow = days of immunization. Full arrow = day of infection.

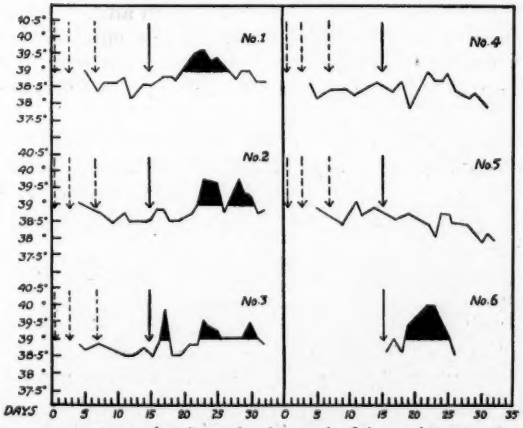

CHART 2B.—Temperature curves of guinea-pigs immunized in various ways with formolized cultures of rat virus. No. 1 received 3 injections of 1/4 c.c. of culture. No. 2 received 3 injections of 1 c.c. supernatant fluid. No. 3 received 3 injections of 2 c.c. supernatant fluid. Note aborted infection. No. 4 received 3 injections of 0·5 c.c. culture suspension. No. 5 received 3 injections of 1 c.c. culture suspension. No. 6 Control.

The injections were given on 10.vi, 13.vi and 17.vi respectively, and the immunity tested on 25.vi, 8 days after the last inoculation.

The results are summarized in Table I. It should be noted that none of the animals showed any rise in temperature during the vaccination, and that adequate controls were used when the animals were tested for immunity. A number of typical charts (Charts 2A and 2B) are given showing the course of the temperature curves during vaccination, and following the injection for testing immunity.

TABLE I.—*Immunization of Guinea-Pigs with Formolized Cultures of* Rickettsia.

Set.	Vaccine material.	Amount injected (c.c.).	Number of injections.	Time interval (days).	Number of animals.	Results of infection.			
						Time after last inoculation (days).	Infection.		
							Positive.	Aborted.	Negative.
A	Formalized supernatant fluid	1·00	3	3	2	10	2	0	0
B	Ditto	2·00	3	3	2	10	0	2	0
C	Saline vaccine	0·25	3	3	5	10	3	2	0
D	,, ,,	0·50	3	3	5	10	0	0	5
E	,, ,,	1·00	3	3	5	10	0	0	5
Control	..	..	..	..	2	..	2	0	0

It is apparent from the results of this experiment that the supernatant fluid of the culture contains a small amount of antigen. The animals which received a total of 3 c.c. manifested no immunity, while those which received 6 c.c. (equivalent to the material of two culture flasks) possessed a partial immunity.

Regarding the *Rickettsia* suspension, it is apparent that a total of 0·75 c.c. of the vaccine was not adequate to induce immunity. Three of the animals succumbed to the infection, while in the other two the infection was modified. Double that amount (1·5 c.c.) appears to contain sufficient antigen to produce a degree of immunity sufficient to protect the animals against the infective dose used.

Immunization of Rabbits.

Breinl (1924), in his studies on the immunizing effect of phenolized suspensions of *Rickettsia* from infected lice, showed that the rabbit was a suitable animal for testing the antigenic property of the vaccine. Treated rabbits developed antibodies for X19 just as they did when injected with virulent brain virus; and, moreover, rabbits so treated did not react to the injection of virulent material by an increase in the titre. We repeated this experiment with our culture vaccine. Four rabbits were used: 2 received 3 injections of 1 c.c., and 2 others of 0·5 c.c., of the formolized culture suspension. Three weeks after the last injection the rabbits were given 1·0 c.c. of a 1 : 100 suspension of virulent brain intraperitoneally. The results are summarized in Table II:

TABLE II.—*Course of Weil-Felix Reaction in Rabbits Immunized with Culture Vaccine and Subsequently Infected with Brain Virus.*

Date of test.	No. of rabbit.																			
	1.					2.					3.					4.				
	Dilutions.																			
	10.	20.	40.	80.	160.	10.	20.	40.	80.	160.	10.	20.	40.	80.	160.	10.	20.	40.	80.	160.
2.vii	+	±	±	..	..	—	—	—	—	..	+	±	—	—	..	+	+	+	±	..
13.vii	++	++	++	++	++	—	—	—	—	..	—	—	—	—	..	±	—	—	—	..
20.vii	++	++	++	+	+	+	±	±	±	—	+	+	±	±	—	+	+	+	+	..
1.viii	++	++	++	+	±	+	+	±	±	—	++	++	+	+	—	+	±	—	—	..
26.viii	++	++	++	++	+	+	±	±	—	—	+	±	—	—	—	±	—	—	—	..

Notes.—The first test was made on 2.vii before the first immunization injection was given. The second test was made on 13.vii, one week after the last injection. On 7.viii the animals were injected with brain virus, and the effect of the injection on the titre determined on 26.viii. Nos. 1 and 2 were given intracutaneous injections 2.vii, 4.vii and 6.vii. Nos. 3 and 4 were given three intravenous injections of the same material at the same time.

It will be noted that the treated rabbits reacted in the same manner as those receiving louse virus vaccine. The injection of vaccine induced a small increase in the agglutinating titre to X19, which remained stationary, or even regressed after the injection of the virulent tissue virus. It is evident, therefore, that the culture suspension has definite antigenic and immunizing properties which correspond with those of the *Rickettsia* suspensions from guts of infected lice.

Immunization of Macacus rhesus.

Lepine (1933) has shown that both *Macacus* and *Cercopithecus* monkeys react to rat virus with an acute febrile disease with the production of a positive Weil-Felix reaction. An intraperitoneal infection is followed, after an incubation period of 6 to 10 days, by a rise in temperature to the neighbourhood of 40° C., lasting 4 to 8 days, and terminating by lysis. During the febrile period the animal refuses food and suffers from marked prostration.

At the moment we had only one normal young monkey (8 months old) suitable for immunization. The monkey received three 1 c.c. injections subcutaneously, on 25.v, 28.v and 1.vi respectively. On 15.vi it received 0·3 c.c. of a virulent tunica suspension (1 : 20). During the immunization the temperature remained normal, the animal was active and ate well. After the infection the temperature fluctuated between 38·5° and 39·0° until 1.vii, when it rose. The temperature during the next few days was as follows: 1.vii, a.m. 39·7° C., p.m. 39·1° C.; 2.vii, a.m. *40·2°* C., p.m. 39·0° C.; 3.vii, a.m. 39·1° C.; 4.vii, a.m. 39·0° C. Thereafter it was 38·5° to 39·0° C. It is likely that the brief rise in temperature was due to skin wounds caused by the belt, because the animal manifested no other symptoms, ate well and showed none of the characteristic signs of depression.

The results of the Weil-Felix test determined at various intervals are of interest. Tests were made on 28.v—after the first injection; on 12.vi—twelve days after the last injection; on 8.vii—three weeks after infection with the tunica virus; and on 2.viii—three weeks after infection with European virus.

The results were as follows :

	1:10.	20.	40.
28.v	±	—	—
12.vi	+	±	—
8.vii	+	±	—
2.viii	+	+	±

It will be noted that, as in the case of the rabbits, the vaccine produced a low rise in titre, which remained practically unchanged after subsequent injection of virulent virus of the rat and European strains respectively. The lack of increase in the X19 titre after infection with the rat virus indicates that the monkey had developed active immunity, and supports the surmise that the short rise in temperature was due to other causes.

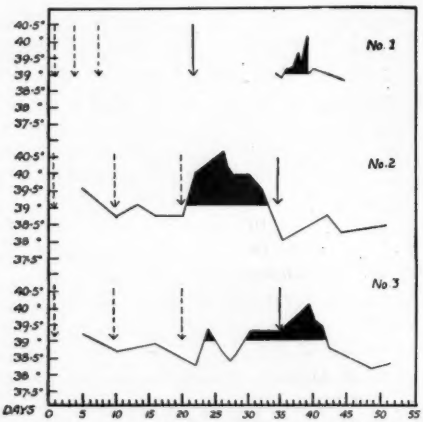

CHART 3.—No. 1 : Temperature curve of monkey infected with rat virus following vaccination with culture vaccine of the rat strain. During vaccination the temperature taken at intervals remained normal. The maximum titre with X19 was 1 : 40. Nos. 2 and 3 : Temperature curves of monkeys immunized with formolized brain virus. The temperatures developed during the immunization, in No. 2 as a result of the second injection, and in No. 3 after the third injection. In both cases the Weil-Felix reaction was positive in a dilution of 1 : 500.

For purpose of comparison it may be of interest to present a protocol of an immunization experiment with formolized brain virus from a guinea-pig infected with European typhus. The monkey received three successive injections of 1·0 c.c. each of a 1 : 50 brain emulsion exposed to 0·1 p.c. formalin. The first injection was given after 2 hours' exposure to formalin, the second after 1 hour and the third after ½ hour. One animal developed a typical infection 10 days after the 2nd injection, while the other developed an aborted infection 12 days after the 3rd injection. The interesting point is the course of the infection, and the X19 titre at the termination. In both cases the Weil-Felix reaction was positive in a dilution of 1 : 500. The course of the infection is shown in Chart 3.

DISCUSSION.

The experiments reported above demonstrate that it is possible to immunize animals with formolized suspensions of *Rickettsia* cultures grown in flasks in the presence of live tunica tissue. These cultures contain large numbers of organisms in and surrounding the tissue material, and smaller numbers in the culture fluid. The emulsions obtained from these cultures constitute as effective antigens as those obtained from the guts of infected lice.

The data also demonstrate that the antigenic behaviour of *Rickettsia* is very much like that of bacteria. The immunity response is (within limits) dependent on the quantity of antigen used. A relatively small amount of antigen, such as is contained in the fluid part of the culture medium, or in only 0·25 c.c. of the culture suspension, is inadequate to produce immunity. We believe that this fact accounts for the failure to produce immunity with killed virus in infected tissue. Infected guinea-pig brain is infective up to a maximum dilution of 1 : 20,000 or 1 : 40,000; in other words, a gramme of brain tissue may be estimated to contain 400,000 to 4,000,000 organisms. The difference between brain tissue and culture or louse gut lies in the fact that in the first the dead organisms are presumably phagocyted and eliminated, whereas in the others they accumulate in the lumen of the gut, or in the culture fluid. The amount of antigen in the last two is, therefore, many times larger than in the brain tissue. This explains why the culture of one-sixth of a tunica or the guts of 12 infected lice induce an effective immunity in the guinea-pig, whereas 3 g. of brain will at best produce only a slight or partial immunity.

These considerations may also be applicable to the problem of immunization with virus. The repeated demonstration of the failure to produce active anti-viral immunity with dead virus may well prove to be purely a matter of quantity of antigen. The difficulty of obtaining a billion or more virus elements in a small volume of fluid may lie at the bottom of this problem. Suggestive confirmation of this hypothesis has been obtained by one of us (Kligler) with vaccine virus; in two instances it was possible to immunize rabbits with large doses of virus killed by heating to 60° C. for 45 minutes. Tissue cultures of viruses may open the possibility of developing dead vaccines for immunization against virus infections.

The similarity in behaviour between *Rickettsia* and viruses with regard to cell parasitism suggests that the basic reason for failure to immunize with dead tissue virus is the same in both. The solution of that problem with regard to *Rickettsia* opens, therefore, the possibility of a solution with regard to virus diseases in general.

CONCLUSIONS.

1. Data are presented showing that it is possible to immunize animals with formolized suspensions of tissue cultures of *Rickettsia*.

2. Cultures of the European and Mediterranean or rat virus were used, and in each instance three injections, equivalent to about one-sixth of a tunica, proved sufficient to induce an effective immunity.

3. Old as well as fresh cultures and freshly formolized suspensions as well as old ones are equally effective for immunization.

4. Failure to induce immunity with dead tissue *Rickettsia* appears to be due to the insufficient amount of antigen present in the infected tissues. It is suggested that this is the plausible reason for a similar failure in the case of viruses, and that vaccines of virus cultures may offer a solution to the problem of active immunization with dead virus.

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OBSERVATIONS ON THE PROPERTIES OF THE VI ANTIGEN OF *B. TYPHOSUS*.

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IN previous papers (Felix and Pitt, 1934*a* and *b*) the existence in strains of *B. typhosus* of a special virulence antigen (Vi) has been described and its immunological significance has been demonstrated. In the present paper observations on some of the properties of the Vi antigen are recorded.

METHODS OF DEMONSTRATING THE VI ANTIGEN BY *IN VITRO* TESTS.

The resistance to O agglutinins of suspensions of live cultures is an indicator of the presence of Vi antigen in strains of *B. typhosus*. The test for the inagglutinability of the live culture is, therefore, the simplest *in vitro* test for virulence. It is, however, an indirect method of demonstrating the Vi antigen, and it was thought possible that the direct method, *i. e.* agglutination by means

of the Vi antibody, might give more accurate results. It has been shown in a previous paper (Felix and Pitt, 1934*b*) that a serum containing Vi antibody alone is readily obtained, if the accompanying O and H antibodies are removed from the serum by absorption with a highly agglutinable strain of *B. typhosus*, which is devoid of Vi antigen. A pure Vi antiserum could thus be used in agglutination tests along with pure O and H antisera, the latter being the serum from a rabbit immunized with a motile and completely rough variant of *B. typhosus*.

TABLE I.—*Agglutination by Pure H, O and Vi Serum.*

Agglutination of Living Organisms.

Serum.	Dilution.	Strains. Ty 2.	Giglioli.	Watson.	b 1.	b 2.	Ty 8.	Ty 901.	Rawlings.
Pure H serum No. 36	1 : 2000	+	++	+	++	+	++	++	+
Pure O serum No. 20	1 : 1000	f. tr.	f. tr.	f. tr.	+	++	+++	+++	+++
	1 : 2000	—	—	—	+	+±	+++	+++	+++
	1 : 5000	—	—	—	±	+	+++	+++	+++
	1 : 10,000	—	—	—	tr.	±	++±	++±	++±
	1 : 20,000	—	—	—	f. tr.	tr.	+	+	+
	1 : 50,000	—	—	—	—	f. tr.	tr.	tr.	tr.
Pure Vi serum No. 82	1 : 200	++±	+++	+++	++±	+±	—	—	—
	1 : 400	+	++±	+++	+±	+	—	—	—
	1 : 800	±	+	++	+	±	—	—	—
Virulence to mice		High			Intermediate		Low		

Table I shows that Vi agglutination and O agglutination are mutually exclusive. The two extreme types of strain are sharply differentiated by both reactions; strains of intermediate virulence hold an intermediate position in the two agglutination reactions.

It has been stated in a previous paper (Felix and Pitt, 1934*b*) that the intensity of the Vi agglutination, exhibited by highly virulent strains of *B. typhosus*, shows a constant relationship to the degree of virulence. It is seen from Table I that the strain "Watson" is most readily agglutinated by the Vi antibody. Next follows the strain "Giglioli" and then "Ty 2". This is the inverse order of their relative virulence. The most plausible explanation of this unexpected phenomenon seemed to be that strains of highest virulence, such as "Ty 2", containing in them the greatest amount of Vi antigen, require for their neutralization the greatest quantity of Vi antibody, and that consequently the titre of the reaction is lowered. It will be observed, however, that this hypothesis is not supported by the results obtained with strains of intermediate virulence (see Table I, strains b 1 and b 2). With this type of strain the degree of virulence cannot be inferred with greater accuracy from the intensity of the Vi agglutination than from that of O agglutination. It is possible that quantitative absorption of Vi antibody would be a reliable indicator of the degree of virulence. However, even this complicated method could not claim the same significance as that to be ascribed to virulence tests in the animal.

From the comparison of the direct and indirect methods of demonstrating the Vi antigen by *in vitro* tests it is evident that the two are confirmatory of one another. The direct demonstration by a pure Vi antiserum is certainly the more impressive test. On the other hand, the test for inagglutinability by a pure O antiserum is simpler to perform and gives equally reliable results.

EFFECT OF GROWTH AT VARIOUS TEMPERATURES.

An observation made in the course of phagocytosis tests to be recorded in another paper led to the investigation of the effect on the development of Vi antigen of growing virulent cultures at various temperatures. Ledingham and Dean (1912), in their early experiments on phagocytosis, used suspensions of *B. typhosus* grown at 20° C., since the bacilli grown at this temperature were of considerably greater size than those resulting from cultures grown at 37° C., and were therefore more readily counted inside leucocytes than bacilli grown at 37° C. When attempting to follow this procedure it was found that our most virulent strains of *B. typhosus*, which were known to us to be highly resistant to phagocytosis when grown at 37° C., lost this property when grown at 20° C. Simultaneously the resistance to O agglutinins was annulled, and the virulence to mice reduced considerably. An illustrative experiment is reproduced in Table II.

TABLE II.—*Effect of Growth at 20° C.*

		Strain Ty 2 grown 24 hours on plain agar at—		Strain Giglioli grown 24 hours on plain agar at—	
		37° C.	20° C.	37° C.	20° C.
Phagocytosis.					
Number of organisms phagocyted by 100 leucocytes		12	78	14	98
Cells taking part p.c.		12	43	14	57
Agglutination.					
Pure O serum, No. 20	1 : 1000	f. tr.	+++	f. tr.	+++
	1 : 2000	—	+++	—	+++
	1 : 5000	—	++	—	+++
	1 : 10,000	—	+±	—	++±
	1 : 20,000	—	±	—	+±
	1 : 50,000	—	tr.	—	±
Pure H serum, No. 36	1 : 2000	+	++	++	++
Virulence to Mice.					
Dose 100 × 10^8 intraperitoneally		8/8	3/8	8/8	2/8

Note.—The technique of the agglutination and virulence tests has been described in a previous paper (Felix and Pitt, 1934*a*).
The technique of the phagocytosis tests will be described in a subsequent paper.

The results recorded in Table II strikingly recall the change in agglutinability and virulence produced by growing virulent strains of *B. typhosus* on phenol agar (see Felix and Pitt, 1934*a*, Table V). The sudden drop in virulence resulting from growth on plain agar at 20° C. for a period so short as 24 hours appears the more impressive, since the first subcultures grown at 20° C. of

necessity contain a considerable proportion of highly virulent organisms, which have been carried over while seeding a loopful from an agar slope grown at 37° C.

Following this observation the development of Vi antigen in cultures grown at various temperatures was studied more systematically. For reasons of economy the cultures were not tested for their virulence to mice. The presence or absence of the Vi antigen was determined by the sensitiveness of the cultures to O agglutination and to phagocytosis. Table III illustrates some of the results obtained in numerous experiments.

TABLE III.—*Effect of Growth at Various Temperatures.*

Agglutination.

		Strain Giglioli grown 4 × 24 hours at—					
		20° C.	25° C.	37° C.	40° C.	42° C.	44·5° C.
Pure O serum, No. 20	1 : 1000	+++	++	f. tr.	f. tr.	±	+++
	1 : 2000	+++	+	—	f. tr.	±	+++
	1 : 5000	+++	tr.	—	f. tr.	±	+++
	1 : 10,000	++±	tr.	—	f. tr.	tr.	+++
	1 : 20,000	+±	f. tr.	—	—	tr.	++
	1 : 50,000	±	—	—	—	f. tr.	+
Pure H serum, No. 36	1 : 2000	++	+	+	±	±	—

Phagocytosis.

Number of organisms phagocyted by 100 leucocytes	..	204	13	..	..	682
Cells taking part p.c.	..	90	13	..	..	96

Agar slopes from the same batch of agar were used for series of subcultures, which were grown at various temperatures. They were transplanted daily for four days, and the suspensions used for agglutination and phagocytosis tests were always prepared from 24-hour cultures. It is seen from Table III that by growing the highly virulent strain "Giglioli" at 20° C. and also at 44·5° C. its resistance to the action of the O antibody was entirely annulled. At 25° C. and 42° C. the suppression of the Vi antigen was partial only, but it was discernible even at 40° C. Strains of the highly agglutinable and avirulent type of *B. typhosus* were always included in these experiments, although they are not shown in the table. With these strains there was in no instance any significant difference in O agglutination or phagocytosis between cultures grown at 37° C. and those grown at the various temperatures under test.

It is necessary briefly to describe some changes in the appearance of the colonies of cultures of *B. typhosus* grown at low temperatures, since these may lead to the erroneous conclusion that a change from the "smooth" to the "rough" form has occurred. The appearance of the colonies on agar plates grown at 20° C. is almost indistinguishable from that of some typical "rough" variants. However, the broth cultures grown at that temperature are of uniform turbidity, while the suspensions from agar cultures are perfectly stable in salt solution and are specifically agglutinated by O antiserum (see Table III). These "rough"-looking colonies are almost entirely composed of those unusually long and thick bacillary forms, arranged in threads and chains,

which have been fully described by Arkwright (1930) as occurring in " medusoid " colonies of *B. typhosus*. Arkwright clearly stated that the antigenic properties of medusoid colonies of *B. typhosus* are the same as those of the normal smooth colony, and he emphasized the fact that the appearance of these colonies may lead to error. The first subculture from these " rough "-looking colonies, when grown at 37° C., invariably yields the typical smooth type in pure growth.

Cultures grown at temperatures between 20° and 27° C. exhibit the same change in the appearance of the colonies. This explains why colonies of *B. typhosus* on agar plates grown at 37° C., which, when inspected immediately after their removal from the incubator, have all the characters of perfect smoothness, tend to change into rough-looking colonies after they have been kept for some time at room temperature.

On the other hand, growth at 44·5° C., which also suppresses the Vi antigen, produces quite different changes in cultures of *B. typhosus*. The colonies are of the normal smooth form, whereas the bacilli are very thick ; some are long, but many are short ; they look swollen, and most of them stain faintly and show vacuoles and nodal staining. Growth at temperatures between 40° and 44·5° C. causes the same changes to appear, though to a lesser extent. It is therefore quite evident that there is no constant relationship between the development of Vi antigen and the appearance of the colonies or the morphology of the organisms. Furthermore the changes in these characters occur in exactly the same manner in highly virulent strains of *B. typhosus* and in those which are entirely devoid of Vi antigen.

The suppression of the Vi antigen is neither complete nor permanent. Cultures of the most virulent and inagglutinable strains which by growth at 20° C. had lost these two properties were still capable of inducing the formation of Vi antibody in the rabbit. Just as suddenly as the development of Vi antigen is suppressed by growth at unsuitable temperatures, so it is re-stimulated by incubation at 37° C. We have grown some of our virulent strains at 20° C. and 44·5° C., subculturing daily for periods of 25 and 15 days, respectively. Immediately on incubating one of these subcultures for 24 hours at 37° C. the inagglutinability by O antiserum was invariably found to have been fully restored, thus indicating abundant development of Vi antigen.

These observations appeared particularly striking in view of the fact, previously emphasized (Felix, 1929), that the degree of agglutinability, which indicates the presence or absence of Vi antigen, is a remarkably constant property of different strains of *B. typhosus*. It has been stated in a previous paper (Felix and Pitt, 1934*a*) that our most virulent strain, Ty 2, was isolated as long ago as 1918. It may be added that to one of the present writers (F.) a number of other strains isolated in 1918 and described in previous papers are known to have maintained their respective agglutinative properties entirely unaltered. In one instance, however, the definite loss of the Vi antigen originally present in a strain of the type of intermediate agglutinability has been observed. The strain Ty 1, extensively used in earlier work (Felix, 1924) now exists in two variants, namely, one of the original intermediate type which contains Vi antigen, and one of the highly agglutinable type, which is entirely devoid of Vi antigen.

The striking effect on the formation of Vi antigen of growing cultures at various temperatures is not only an interesting phenomenon but also one of practical importance. In experimental work concerning the Vi antigen or the virulence of *B. typhosus* the constancy of the temperature at which the cultures are grown must be carefully watched. It is also essential to reduce to a minimum the time during which the cultures are manipulated at room temperature.

It is suggested that the facts established in experiments with *B. typhosus* might usefully be applied to similar studies in other bacterial species.

THE HEAT RESISTANCE OF THE Vi ANTIGEN.

It has been shown in a previous paper (Felix and Pitt, 1934*a*) that the inagglutinability by O antiserum of virulent strains of *B. typhosus* is suppressed by heating for one hour at 60° or 100° C. It was also found (Felix and Pitt, 1934*b*) that only sera prepared by immunizing rabbits with suspensions of living virulent bacilli contained Vi antibody, whereas suspensions heated to 58°, 70° or 100° C. were incapable of inducing the formation of this antibody. On the other hand, suspensions heated to 58° C. were found to be capable of inducing active immunity in the mouse. This apparent discrepancy called for a more detailed investigation of the heat resistance of the Vi antigen.

Re-examination of a number of rabbit sera, which had been prepared by intravenous inoculation of suspensions heated for one hour at 60° C., disclosed the presence of Vi antibody in the serum of almost all the rabbits which had been immunized with the virulent and also with the intermediate type of *B. typhosus*. However, the Vi titres of these sera were extremely low, not exceeding a dilution of 1 in 10, and this antibody was usually found to have made its appearance in the serum only after prolonged hyper-immunization. To give an example, serum drawn after a series of four intravenous inoculations, in the course of which a total of $12{,}000 \times 10^6$ organisms had been injected, was usually found to be still devoid of Vi antibody. After further inoculation of a dose consisting of 6000×10^6 organisms the antibody was found to be present in serum dilutions of 1 in 5 or 1 in 10.

The normal sera of all the rabbits used for immunization, which were taken before the injections were begun, were examined simultaneously as controls. Distinct Vi agglutination could be observed with some of these sera in dilutions of 1 in 5 and even 1 in 10. In common with all the other known immune bodies, the Vi antibody obviously possesses its prototype in a natural Vi antibody occurring in the serum of normal animals. In the serum of some normal horses the natural Vi antibody was found, even in a dilution of 1 in 20. Neither in the rabbit nor in the horse sera did those giving the highest titres of the natural Vi antibody always coincide with the specimens giving the highest titres of the natural O antibody.

The agglutinability by pure Vi antiserum of suspensions heated at various temperatures was then investigated. Table IV gives an illustrative experiment.

Table IV shows that the Vi antigen remains demonstrable by agglutination tests after heating to 58° C. for ½ hour and even for 1½ hours, and also after ½ hour at 60° C. After heating to 60° C. for 1 hour or to 100° C. for 10

minutes the Vi antigen is no longer demonstrable by agglutination. Absorption tests, however, clearly indicated that the Vi antigen withstood exposure even to 100° C. Suspensions steamed at 100° C. for 1 hour were still capable of specifically absorbing Vi antibodies, though their absorbing power was much lower than that of unheated suspensions.

TABLE IV.—*Heat Resistance of the Vi Antigen Demonstrated by Vi and O Agglutination.*

Strain: Watson. Saline suspensions from agar slopes.

Serum.	Dilution.	Fresh, living.	Heated at 58° C.		Heated at 60° C.		Heated at 100° C. 10 minutes.
			½ hour.	1½ hours.	½ hour.	1 hour.	
Pure O serum, No. 20	1 : 1000	f. tr.	++	++±	++	+++	+++
	1 : 2000	—	++	++±	++	+++	+++
	1 : 5000	—	+±	++	+±	++	++
	1 : 10,000	—	+	+	+	+	+
	1 : 20,000	—	±	±	±	±	±
	1 : 50,000	—	—	—	—	—	—
Pure Vi serum, No. 82	1 : 200	+++	++±	tr.	+±	—	—
	1 : 400	+++	+	f. tr.	+	—	—
	1 : 800	++	+	—	±	—	—
Pure H serum, No. 36	1 : 2000	+	+	+	+	+	—

It is well known that the various *in vitro* and *in vivo* activities of an antigen differ widely in their capacity to demonstrate the degree of deterioration of antigen due to heating or treatment with various chemicals. This is also the case with the Vi antigen. The discrepancies between the results of active and passive immunization and those of the *in vitro* tests are apparent only, and due to the different degrees of fineness exhibited by the various reactions.

METHODS OF PREPARING Vi ANTISERA.

The conclusion was reached from previous experiments (Felix and Pitt, 1934*b*) that Vi antisera could not be obtained by immunizing rabbits with suspensions of heat-killed virulent bacilli, but only by the use of the living organisms. Even by this procedure only Vi antisera of comparatively low titres were obtained. In the first series of four rabbits so immunized the highest titre of agglutination due to the Vi antibody did not exceed a dilution of 1 in 400.

From the experiments described in the present paper it is evident that suspensions of organisms killed by heating to 60° C. for 1 hour, though capable of inducing the formation of a certain amount of Vi antibodies, are quite unsuitable for the preparations of potent Vi antisera. On the other hand, the immunization of rabbits with virulent bacilli in the living state entails considerable risks. Attempts were therefore made to avoid this obvious disadvantage by the use of an antigen sterilized in such a way as to preserve its capacity for stimulating efficient formation of the Vi antibody.

Saline extracts from living virulent organisms proved to be serviceable. They were prepared in the following way: Agar cultures grown in Roux bottles for 24 hours at 37° C. were suspended in saline, using 12 c.c. of saline

for the growth from one Roux bottle. The suspensions, which usually contained 160,000 to 200,000 million organisms per c.c., were left for 2 hours at 37° C. and then centrifuged; the supernatant fluid was removed and sterilized by the addition of 0·2 p.c. formalin. After 24 hours at room temperature these extracts were invariably found to be sterile. They were then stored in the cold room.

Precipitation tests with extracts from virulent and avirulent strains of *B. typhosus* showed that the former possessed both Vi and O antigen, while the latter contained O antigen only. This is illustrated in Table V.

TABLE V.—*Precipitation Tests.*

Saline extracts from strains.	Pure Vi serum No. 62. 0·05 c.c. of dilution 1 in 5. 0·2 c.c. of extract in dilution.				Pure O serum No. 20. 0·05 c.c. of dilution 1 in 5000.	Type of strain.
	1 : 1.	1 : 5.	1 : 20.	1 : 200.	1 : 5.	
Ty 2	tr.	+	±	—	+++	Virulence high.
Giglioli	+	++	±	—	+++	
Watson	±	+±	±	—	+++	
Ty 8	—	—	—	—	+++	Virulence low.
Ty 901	—	—	—	—	+++	
Rawlings	—	—	—	—	+++	

Reading with the naked eye after 24 hours (3 hours at 37° C., then in the cold room).

The precipitate resulting from the interaction between the Vi antigen and the Vi antibody resembled in its appearance that formed by the O antigen and its corresponding antibody; the latter, however, was much more abundant.

Extracts from cultures of the virulent type of *B. typhosus* were also tested for their capacity to inhibit the agglutination reaction between sensitive strains and O serum. No inhibition of O agglutination was observed, though it must be admitted that these attempts were not pursued sufficiently to establish optimal conditions for this interaction. In these agglutination experiments, as well as in the precipitation tests, the extracts used were free from formalin.

Rabbits immunized by intravenous inoculation of formolized extracts from virulent strains of *B. typhosus* responded with relatively abundant formation of Vi antibodies. In a series of three rabbits the titres of Vi agglutination observed were: 1 in 200, 1 in 600 and 1 in 800 respectively. These titres compare very favourably with those obtained in a series of four rabbits immunized with living virulent bacilli (Felix and Pitt, 1934*b*), where the corresponding figures were: 1 in 50, 1 in 100, 1 in 200 and 1 in 400. Prolonged hyperimmunization with the extracts was required to produce this effect. The high toxicity for rabbits of extracts of *B. typhosus* is well known (see White, 1929). It is therefore advisable to use for the intravenous inoculations only extracts diluted with saline. Taking this precaution we did not lose any of the rabbits which were given four or five inoculations at intervals of two days, the first dose consisting of 0·1 c.c. of extract in 2 c.c. of saline, and the last dose containing 1·5 c.c. of extract diluted with 3 c.c. of saline.

Extracts were also prepared by repeatedly freezing and thawing the bacillary suspension. No advantage resulted from this procedure. It was also attempted

to sterilize the extracts by filtration through Chamberland L5 candles. However, filtration was found to cause considerable loss in the potency of the extracts and was, therefore, abandoned. Since the presence of formalin in extracts containing the Vi antigen does not destroy its agglutinogenic activity, it seems very probable that formolized suspensions of the whole culture of virulent bacilli could also serve as efficient antigens.

SUMMARY.

1. The Vi antigen of *B. typhosus* can be demonstrated by the inagglutinability of the living organisms by pure O serum or by agglutination with pure Vi serum. The two methods give equally reliable results.

2. The development of Vi antigen is suppressed by growing virulent strains of *B. typhosus* at temperatures between 20° and 25° C., and also between 40° and 44·5° C. The application of this technique to similar studies in other bacterial species is suggested.

3. The heat resistance of the Vi antigen is described as it is reflected by agglutination and absorption tests and by antibody formation in the rabbit.

4. Saline extracts of cultures of virulent strains of *B. typhosus* contain Vi antigen precipitable by pure Vi antiserum.

5. The use of formolized extracts is suggested for the preparation of relatively potent Vi antiserum.

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ANTIGENIC DIFFERENCES BETWEEN RELATED BACTERIAL STRAINS: A CRITICISM OF THE MOSAIC HYPOTHESIS.

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SINCE it was first put forward by Durham (1901) the mosaic conception of bacterial antigens has been accepted by all bacteriologists as at least a useful convention, and by most as a correct picture of antigenic structure. The usual technique of cross-absorption tests, as applied for instance to the study of the Flexner dysentery bacilli, shows clearly that a given serum can be divided by suitable absorptions into fractions of quite different agglutinating power. It is natural to assume that these demonstrable differences amongst

antibody molecules must be directly related to corresponding differences amongst the antigen molecules forming the sum of the antigens of the bacterium. To take a concrete example, an antiserum produced against a Y (Hiss and Russell) strain will agglutinate V, W and Z strains to considerable titres, and by suitable absorptions this serum can be made to agglutinate any one of V, W or Z without more than a minimal effect on the other two types. Since V, W and Z strains and antisera show little cross-agglutination amongst themselves, the hypothesis was put forward by Andrewes and Inman (1919) that the Y strain was antigenically a mosaic of V, W and Z antigens.

In studying the phage-inhibiting activity of bacterial extracts, the nature of cross-reactions, both immunological and concerning bacteriophages, presented continual problems. It seemed possible, once it had been shown that phage-inhibiting activity is wholly a function of the bacterial (polysaccharide) antigen, that a direct decision on the correctness or otherwise of the mosaic hypothesis could be reached by means of this technique. In brief, if the antigenic structure of Y is a mosaic of V, W and Z antigens, then a filtered solution of Y antigen should be immunologically indistinguishable from a suitable mixture of V, W and Z antigen solutions. Thus if a Y extract is treated with a pure W antiserum, only a portion of the antigen should be removed from solution, the V and Z components being unaffected, as they would be in a mixture. Such a problem might be studied on purely immunological lines, but a much more convenient and delicate method is available in the technique of phage inhibition as described by Burnet and Gough (1934). Suitable extracts were prepared, containing considerable amounts of polysaccharide in the active phage-inhibiting condition from each type of bacillus, and agglutinating sera were produced by the ordinary method of immunization with formalin-killed broth cultures. Precipitin reactions occurred between the various sera and extracts in close correspondence with their cross-agglutinating reactions. When the precipitate had been removed by centrifugation the supernatant fluid was titrated for its phage-inhibiting capacity against suitable phages, and the titre compared with the same extract mixed with a R dysentery antiserum which had no visible precipitating power, but might be expected to react with any components of the extracts other than the specific smooth polysaccharides. The results showed conclusively that when an extract gave a well-marked precipitation with any serum the whole of the phage-inhibiting capacity was removed. A Y extract could be exhausted of practically all its activity by V, W or Z antisera, but a mixture of Z and W extracts treated with W serum lost only the activity due to the W extract.

EXPERIMENTAL.

The reagents employed were derived from the classical strains of Andrewes and Inman (1919), W (Cable), Y (Hiss and Russell) and Z (Whittington). Antisera were made in the usual fashion, and their agglutination titres against these three organisms were as shown in Table I.

Extracts were prepared by adding 10 g. of urea to 10 c.c. of a thick emulsion of organisms washed off from 15–20 Petri dishes of 24-hours' growth. This was warmed for a few minutes to 50° C. at a reaction adjusted to approximately

pH 7·0 and gave a viscous half-dissolved mass. About 1 c.c. of 10 p.c. acetic acid was added, giving a reaction around pH 4·5, and the material was then dialysed through cellophane for 24 hours. The dense precipitate which formed was spun off after adjustment, if necessary, to pH 4·5. The supernatants were usually only faintly opalescent, and could be filtered through an Elford membrane filter with only a small loss of activity. In the present experiment the Y extract used was filtered, but W and Z extracts, which were of lower titre, were used without filtration after prolonged high-speed centrifugation.

Three phages which were relatively easily inactivated by suitable extracts were used: H and L (of Morison's Indian series of dysentery phages), and D6 (Burnet and McKie, 1930). The activity of these phages on the different strains and the phage-inhibiting activity of the extracts on each are shown in Table II. The phage-inhibiting activity (P.I.A.) is in terms of the final dilution of extract capable of reducing the phage titre to 20 p.c. of its original value after 4 hours at 50° C., or in the case of D6 at 39° C. because this phage is inactivated fairly rapidly at 50°.

TABLE I.—*Agglutination of W, Y and Z Strains of Flexner Bacilli.*

Antiserum.	Titre against—		
	W.	Y.	Z.
W	3600	6400	100
Y	800	12,800	1200
Z	160	10,000	6400

TABLE II.

Phage.	Lytic action on—			P.I.A. of extracts.		
	W.	Y.	Z.	W.	Y.	Z.
H	+	+	+	20	2000	400
L	+	+	—	25	30	0
D6	—	+	+	0	10,000	10,000

TABLE III.—*Influence of W, Y, Z and R Sera on Active Extracts of Y and Z Strains.*

Sera.	Extract Y.			Extract Z.	
	Precipitation.	Residual P.I.A.		Precipitation.	Residual P.I.A.
		Phage H.	Phage L.		Phage H.
W	+++	1	0	tr.	400
Y	+++	1	0	+++	3·5
Z	+++	1	0	+++	<1
R (control)	—	1900	30	—	600

The figures shown under P.I.A. (phage-inhibiting activity) represent the approximate final dilution of extract which under the standard conditions of experiment would reduce the titre of the phage in question to 20 p.c. of the original. In this experiment these values indicate the relative amounts of antigen remaining unprecipitated in the serum-extract mixtures.

Behaviour of Y and Z Extracts with Unabsorbed Sera.

Mixtures were made of equal volumes of extract and serum; these were incubated for an hour at 50° C. and then placed in the refrigerator. The following day the tubes were centrifuged and the supernatants titrated as previously described against phages H and L.

The degree of precipitation and the residual phage-inhibiting titres were as shown in Table III.

All but a trace of activity is removed from Y extract by any of the three sera which precipitate with it.

Comparison of a Y Extract with a Mixture of W and Z Extracts.

In this experiment a W serum was used which had been freed from its slight action on Z extracts by absorption with washed Z bacteria. Controls showed that only a trace of Z phage-inhibiting activity was conferred on the serum by this procedure. Against the highly sensitive phage D6 the serum alone showed a phage-inhibiting activity of 2 as against a value of approximately 10,000 for the Z extract used. The same R serum was used as a control. In the preliminary precipitation one volume of each extract was mixed with two of serum, an additional volume of broth being added to the tubes containing only one extract. The results of titrations of the supernatants are expressed in Table IV.

TABLE IV.—*Influence of W Serum on the P.I.A. of Y, W, Z and mixed W and Z Extracts.*

Sera.	Y extract.		W extract.		Z extract.		W and Z extracts.		
	Phage H.	Phage D6.	H.	L.	H.	D6.	H.	L.	D6.
R (control)	2500	10,000	25	25	400	10,000	350	25	10,000
W	0	0	0	<1	500	8000	400	<1	10,000

The figures express the approximate phage-inhibiting activity of the supernatant fluids from serum-extract mixtures against the phages shown.

Table IV brings out particularly clearly the difference between Y extract and a mixture of W and Z, from which only the W component inhibiting phage L is removed by the W serum.

DISCUSSION.

The problem of the nature of group reactions has occupied a number of bacteriologists, but the only authors to describe experiments along lines at all similar to those of the present communication are Furth and Landsteiner (1927). They were studying the polysaccharide haptenes of the Salmonellas, and confirmed the conclusion of Bruce White (1926) that these haptenes corresponded in their specificity with the heat-stable (O) antigens and were unrelated to the H antigens. They endeavoured to obtain evidence as to whether the composite nature of certain O antigens postulated by Bruce White from cross-absorption tests could be directly demonstrated in corresponding bacterial extracts. Their method was to precipitate the extract,

presumably complex, with a serum from which all but one antibody factor had been removed by appropriate absorptions with heterologous bacteria. The precipitate was washed and dissociated by a few seconds' boiling in weak alkali, and the re-liberated haptene examined for its precipitating power with various sera. In each instance its reactions were practically the same as those of the original extract, not those which would correspond to the hypothetical single factor which should have been isolated by the absorbed serum used. On the other hand, artificial mixtures of haptenes of different origin could be readily separated by the same technique.

Such results correspond entirely with those obtained in the present work with Flexner dysentery bacilli extracts. They may be summarized as indicating that while a given antiserum corresponding to a bacterial polysaccharide antigen can be readily fractionated by immunological methods into dissimilar parts, no such components can be demonstrated for the antigen. This asymmetrical relationship between antigen and antibody is precisely what would be expected from such a conception of the antigen-antibody reaction as has recently been developed by Marrack (1934), largely on the basis of the work of Landsteiner and his colleagues. According to this view, " antibody " consists of globulin molecules modified so that certain " active areas " of their surfaces are capable of spatial co-adaptation to and union with certain relatively small " determinant groups " of the antigen molecule. If, as is probably always the case with bacterial polysaccharide antigens, the molecule is large and presents a number of different determinant groups capable of provoking and reacting with specific active areas on globulin molecules, we have at once the probability that the population of modified globulin molecules in the antiserum will not be uniform. If, for example, the antigen molecule possesses four different atomic groupings capable of functioning as determinant groups, A, B, C and D, then in six consecutive antibody molecules we might find corresponding active areas (a b c and d) distributed a a a — a b c d — a a d — b c — b b d — d. Any of these molecules will be capable of union with the homologous antigen, but with a heterologous antigen containing only the determinant group A in common three of the six globulin molecules will remain unattached. On the other hand, if we act on the antigen A B C D with heterologous antiserum, of which a reasonably large proportion of the globulin molecules have an active area " a ", even if there is no other common component the whole of the antigen may be precipitated almost as completely as by the homologous antiserum.

This point of view is almost identical with the second of the alternative explanations offered for cross-reactions in a recent paper by Heidelberger and Kendall (1934) on the reaction between egg albumin and antiserum made against a compound of egg albumin with dye. They consider it simpler, however, to regard " anti-dye " as a single entity, all the characteristic determinant groups being (presumably) regarded as represented on each modified globulin molecule. The difference between heterologous and homologous reactions would then depend on the existence of a high dissociation constant for the former and a low dissociation constant for the homologous reaction. On the view adopted in the present discussion the higher grade of dissociation in heterologous reactions would be accounted for somewhat as follows : Marrack

and Smith (1932) have shown that simple haptene (with a single determinant group per molecule) is readily dissociated from antibody, and does not precipitate. For precipitation to occur some form of lattice structure must develop, each antigen molecule uniting with several antibody molecules and each antibody with several antigen molecules. If this is so it is clear that the greater the number of reciprocally reacting groups which the two reagents have in common, the firmer will be the union and the more complete the precipitation.

In a previous paper (Burnet, 1934) certain evidence was put forward that the antigen molecules in a given extract did not form a uniform population. With one phage it was possible to " absorb " the fraction of the extract responsible for inhibiting this phage without affecting significantly its action on another phage. This apparent fractionation does not conflict with the view here put forward as to the unitary nature of the antigenic pattern of the " whole antigen " molecule. It was shown that the antigenic pattern is much more resistant to such influences as heating with weak alkali than the phage-inhibiting aspects of the molecule, and it is reasonable to expect that in any actual extract only a proportion of the antigen molecules possess the whole of the labile aspects concerned in inhibiting the various phages, although all according to the present evidence carry the full antigenic pattern.

CONCLUSION.

A Flexner dysentery bacillus extract is composed of immunologically similar antigen molecules, and not of the mixture of antigens postulated according to the mosaic theory of bacterial antigenic structure.

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A NEW AGENT FOR STIMULATING THE METABOLISM: DINITRO-O-CYCLOPENTYLPHENOL (PREP. 2769/1) AND ITS ACTION ON THE BASAL METABOLIC RATE.

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For the last few years many new agents have been investigated as regards their action on metabolism and hyperpyrexia. Cazeneuve and Lépine (1885) seem to have been the first scientists who drew attention to the action of certain nitrophenols on the metabolism. They showed that, when injected in small and repeated doses, death occurred with hyperpyrexia. Then, during the war, curious cases of poisoning and death among workers of war munitions happened in France, especially with those connected with the manufacture of dinitrophenols. This led to several researches. A complete report on the physiological and pharmacological properties of dinitrophenol 1.2.4 was published by A. Magne, Prof. A. Mayer and L. Plantefol and their collaborators in 1932. Clinical reports on the same subject were published by Cutting, W. C., Mehrtens, H. G., and Tainter (1933*a*, 1933*b*). These relate the results obtained with the drug in the treatment of obesity.

Dodds and Pope (1933) reported the action of dinitro-o-cresol 4.6 as a new stimulating agent of the metabolism. Other papers (Dodds and Robertson, 1933 ; Dodds and Greville, 1933) record further experience with this new and more active compound.

Thus, both dinitrophenol 1.2.4 and dinitro-o-cresol 4.6 have been well studied and clinically tried. Good results were obtained, but at the same time some unfortunate by-effects were noticed.

The New Compound.

Recently, and at the request of Prof. C. Heymans, of Ghent, who has investigated the problem of these hyperthermic agents since 1928, new intro-derivatives were manufactured. Among them the 2.4-dinitro-o-cyclopentyl-phenol or prep. 2769/1 was found to be the most active. Injected intravenously to the pigeon in the form of the sodium salt in aqueous solution, the dose of 4 to 5 mg. per kg. weight gave rise to an increase of the metabolism such that the temperature rose to 48° C. in 15 minutes. With the dog, the highest stimulation is attained with doses of 7 to 10 mg. per kg. weight.

The general toxicity of this product according to C. Heymans and H. Casier is very low. They report no detrimental action on the respiratory, circulatory and nervous functions of the animals, and come to the conclusion that, so far, this substance is the most active and the most specific on the

cellular metabolism. Compared with the activity of other products they give the following table :

Products.	Relative activity.
Dinitroaminophenol . . .	16·4
Dinitronaphtol	99·3
Dinitrophenol	100·0
Dinitrothymol	178·2
Dinitrocresol	207·5
Dinitrohexylphenol (No. 2755) .	395·1
Dinitropentylphenol (No. 2769) .	455·5

Thus the preparation No. 2769/1 was 4½ times the potency of dinitrophenol.

Such a favourable report induced me to investigate this product on the basal metabolic rate of human beings. In this preliminary report the action of very small doses only has been studied. This research has been carried out in private practice on three female patients.

The New Compound and its Mode of Administration.*

2.4-dinitro-o-cyclopentylphenol in the form of its sodium salt is an orange crystalline powder, partly soluble in water. For administration to patients the drug was mixed with lactose and put in gelatine capsules, each containing 50 mg. of active substance. One or more capsules were taken with a little water after breakfast and after lunch.

Action on the Basal Metabolic Rate.

This action was measured with the British Benedict metabolism apparatus under basal metabolic conditions. The B.M.R. was influenced as follows :

Case 1.—Woman, aged 60 years ; weight 80 kg., with no pathological trouble. The normal B.M.R. is minus 17 p.c. With 1 capsule of 50 mg. daily, for 2 days, or 0·625 mg. per kg. weight and per 24 hours, the B.M.R. rose to minus 9·3 p.c. With 2 capsules daily taken for 2 days, or 1·250 mg. per kg. weight and per 24 hours, the B.M.R. was increased to minus 3·8 p.c. With 3 capsules daily, taken for 2 days, or 1·875 mg. per kg. weight and per 24 hours, the B.M.R. reached plus 2 p.c. With 4 capsules daily, for another 2 days, or 2·5 mg. per kg. weight and per 24 hours, the B.M.R. rose to plus 7·4 p.c. So from the start, the increase obtained in the B.M.R. was slightly over 24 p.c. No untoward symptoms were noticed or reported by the patient.

Case 2.—Woman, aged 25 years, with no pathological trouble ; weight 66 kg. Her normal B.M.R. is minus 3·5 p.c. With 2 capsules daily, or 1·51 mg. per kg. weight and per 24 hours, the B.M.R. was found to be minus 0·2 p.c. after 24 hours. The patient then took 3 capsules daily, or 2·272 mg. per kg. weight, and 24 hours later the B.M.R. was plus 8 p.c. The net increase was thus 11·5 p.c.

Case 3.—Woman, aged 22 years ; weight 49 kg., with no pathological trouble. Her normal B.M.R. is plus 0·5 p.c. With 2 capsules taken for one day, or 2·04 mg. per kg. weight and for 24 hours, the B.M.R. went to plus 28 p.c., after 24 hours, showing an increase of 27·5 p.c.

The last two patients, like the first one, reported no unpleasant reactions. During the tests no respiratory, circulatory, digestive or nervous troubles were noticed. The routine examination of the urine before, during and after the tests has shown no abnormality. The diazo-reaction was positive in the urine of patients taking this polynitrophenol.

* This new compound is manufactured in the laboratories of F. Hoffmann La Roche, S.A: Basle.

Conclusions.

The conclusions to draw from these preliminary tests are the following :

1. The 2.4-dinitro-o-cyclopentylphenol in the form of its sodium salt is an active stimulating agent of the metabolism.

2. Very small doses (from 1 to 2½ mg. per kg. weight and per 24 hours) can stimulate the B.M.R. to about 25–30 p.c. above its normal level (Cases 1 and 3).

3. This increase is brought about without any unpleasant reactions.

The practical results in using this new compound for the treatment of obesity of exogenous origin and certain states of depressed metabolism will be reported on later.

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THE ACTION OF HUMAN BLOOD ON THE MENINGOCOCCUS.

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KOLMER (1918) demonstrated variations in the bactericidal effect of animals' blood, and also showed that the whole blood of young children, in general, was less bactericidal than that of adults. Heist and his colleagues (1922) tested the blood of adults for its action on various strains of meningococcus and demonstrated bactericidal properties in the majority. These results constitute a basis of approach to the study of human immunity and susceptibility to meningococcus infection. We have reinvestigated and extended this work, and correlated our findings with certain biological differences observed in different strains.

TECHNIQUE.

The method used in setting up a test was as follows : Beef infusion agar slopes containing 1 p.c. dextrose and 10–15 p.c. citrated sheep's blood were used as a culture medium. A meningococcus culture recently isolated from

blood or from cerebro-spinal fluid of patients was planted on one dextrose blood-agar slope in a 6 by $\frac{3}{4}$ in. test-tube. After incubation at 36·5° C. for 18–24 hours the entire growth was scraped off the slope with a triangular glass scraper and emulsified in 2 c.c. of normal saline. Such a suspension when diluted 1 : 10 gives a turbidity of approximately 1000 parts per million in terms of the silica standard (Butterfield and Neill, 1920). Tests were at once set up by adding 1 c.c. of fresh, sterile, whole human blood to 0·1 c.c. of the following dilutions of the meningococcus suspension:—Undiluted, 1 : 10, 1 : 100, 1 : 1000, 1 : 10,000, 1 : 1,000,000, and occasionally 1 : 10,000,000. The tubes were immediately shaken to mix organisms with the blood before clotting took place. The tubes were then incubated for 24–36 hours. Samples, taken by breaking up the clots with a strong wire loop, were then planted on dextrose blood-agar plates, incubated for 18–24 hours and examined for the presence or absence of growth. As a control of the viability of the suspended meningococci, a loopful of each of the two highest dilutions of culture was planted on dextrose blood-agar plates. Recently we have modified this procedure in three particulars, that is, by scraping the growth from dextrose blood-agar after 6–12 hours' incubation; by suspending it in broth containing 7 p.c. horse-serum and 1 p.c. dextrose, as suggested by Heist, and by reducing the amount of blood (citrated) to be tested to 0·2 c.c. and adding the blood to 0·01 c.c. of the various dilutions.

EXPERIMENTAL.

A total of 50 samples of human blood have been tested: 33 from normal adults, including non-contact carriers; 11 from infants (2 weeks to 2 years of age), admitted to hospital with various clinical conditions; 2 from infants, 6 months of age, with meningococcal meningitis, blood being taken before and after the administration of serum; 3 from older hospitalized children, 3, 9 and 11 years of age; and 1 from a serum-treated patient 4 years of age, 6 months after recovery from meningitis. Each test was controlled by including in the experiment the blood of an individual known to possess bactericidal power as well as blood manifesting none. The term "bactericidal" is used for convenience of expression and with reservations.

Table I is representative of the results obtained, using a freshly isolated and daily subcultured blood-culture strain from a fulminating case of meningococcal septicæmia.

These tests have been repeated with several samples of adult blood and a few specimens of infants' blood. A recently isolated cerebro-spinal fluid strain was used, and results similar to those illustrated above were obtained. The results suggest that the majority of blood samples from adults, whether carriers or not, are bactericidal to a high degree. Among samples from infants, 11 were non-bactericidal; 2 showed a change from non-bactericidal before, to bactericidal 2 weeks after, the administration of serum; 1 showed bactericidal action 6 months after an attack of meningitis, though a previous test of his blood had not been made. The above findings suggest a possible explanation of the sporadic occurrence of meningitis in infants and young children and its occasional occurrence in adults. This test may afford a possible means of testing an individual's immunity or susceptibility to meningococcus infection.

TABLE I.—*The Action of Human Blood on the Meningococcus.*

Human blood tested.	Undiluted.	Dilutions of meningococcal suspension. 1 in—				
		10.	10^2.	10^3.	10^4.	10^5.
9 normal adults . .	+	+	+	0	0	0
8 normal adults (all non-contact carriers) .	+	+	+	0	0	0
6 normal adults (2 non-contact carriers) .	+	+	+	+	0	0
8 normal adults . .	+	+	+	+	+	+
2 normal adults (non-contact carriers) .	+	+	+	+	+	+
11 infants (2 wks.–2 yrs.)	+	+	+	+	+	+
1 child (3 years) . .	+	+	+	+	+	+
1 child (9 years) . .	+	+	+	0	0	0
1 child (11 years). .	+	0	0	0	0	0
2 infants with meningitis						
Before serum . .	+	+	+	+	+	+
After serum . .	+	0	0	0	0	0
1 child with meningitis (4 years) after serum.	+	0	0	0	0	0

+ = Confluent growth. 0 = No growth.

An experiment was set up to test the effect of a sample of blood from a non-contact carrier on her own strain, 3 freshly isolated strains and 2 old strains. Table II illustrates the results obtained.

TABLE II.—*Action of Sample of Blood on Strains of Meningococci.*

Blood.	Source of strains.	Undiluted.	Dilutions of meningococcal suspension. 1 in—	
			10^4.	10^5.
J. C. .	Ca. C.S.F. .	+	+	+
	X. Ca. N.P. .	+	+	+
	C.C. N.P. .	+	+	+
	J.C. N.P. .	+	0	0
	I—331 .	+	0	0
	II—173 .	+	0	0

From the above experiment it will be observed that a freshly isolated cerebro-spinal fluid strain (Ca. C.S.F.) from a patient, and two freshly isolated nasopharyngeal strains, one from the patient's father (X. Ca. N.P.) and one from a non-contact carrier (C.C. N.P.), grew readily in the sample of blood; whereas the carrier's own strain (J.C. N.P.) and two old stock strains grew only in the undiluted suspension. Upon further investigation it was found that the blood of carrier C.C., whose strain is designated C.C. N.P. in the table, was highly bactericidal to his own strain.

Nine non-contact carrier strains have been tested, in the manner outlined, with a sample of blood which did not exert a bactericidal action upon the

rapidly subcultured cerebro-spinal strain. These strains fell, more or less sharply, into two groups. Six were killed by contact with the blood, except in the case of the two lowest dilutions of bacterial suspension. Three were much more resistant, being only slightly less resistant than the cerebro-spinal strain used as a standard of comparison. Two of these three strains were also tested with the blood of the carriers from whom they were respectively isolated. The carrier blood in each instance was bactericidal to its respective strain.

It was noted that the strains growing in the high dilutions under the conditions of the experiment produced a uniform turbidity in the diluting broth when this was incubated overnight, and that large moist colonies were formed on dextrose blood-agar plates. The three strains upon which the blood exerted a marked bactericidal effect produced, in contrast, a granular sediment in broth with a clear supernatant and smaller colonies on plates. These observations, although not entirely uniform with all strains of meningococcus tested, seem, however, to indicate that freshly isolated strains surviving in the higher dilutions, and resisting the bactericidal action of blood and old stock strains, surviving only in lower dilutions, manifest in general these biological differences. Rake (1933) has drawn attention to these cultural characteristics in meningococci, and correlated them with their ability to grow in rabbits' blood. It is thus evident that among strains of meningococcus there is a wide variation in their ability to survive in any given sample of blood. The possibility that freshly isolated strains from acute cases of meningococcal meningitis *may* show this variation after artificial cultivation is a matter of some importance. The following experience with such a strain is pertinent. The blood-culture strain upon which the reported results of 50 samples of blood were obtained, and which had been daily subcultured up to this time, was lost during a temporary discontinuance of the work. A stock culture of this same strain carried on an egg medium and not subjected to rapid transfer, when used in similar experiments, proved to be entirely unsuitable. Samples of blood known to be non-bactericidal upon the rapidly subcultured strain now killed the suspensions of this stock strain. Up to the present we have found that this change may be prevented if daily subculture is made on dextrose blood agar. A cerebro-spinal strain subcultured daily has preserved its characteristics for a period of some months.

The rapidity of the bactericidal effect of certain samples of blood on a freshly isolated cerebro-spinal fluid strain of meningococcus is presented in Table III.

TABLE III.—*Time Factor in Bactericidal Effect.*

Blood.	Strain.	Hour.	Undiluted.	Dilutions. 1 in—			
				10.	10^2.	10^4.	10^6.
"S"	C. (C.S.F.)	½	+	+	+	13	8
		3	+	+	3	0	0
		5	+	+	16	0	0
		17	+	+	+	0	0
		24	+	+	+	0	0
		36	+	+	+	0	0

Figure = Number of colonies. + = Confluent. 0 = No growth.

This experiment and others performed with different specimens of blood show that the bactericidal effect is quite rapid. The suspension of meningococcus is partially affected as early as ½ hour after the blood is added, and the process is complete in 3 hours. At the end of 4 days a bacteriostatic effect is still maintained.

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THE PRODUCTION OF TUMOURS IN THE FOWL WITH A COLLOIDAL SOLUTION OF 1 : 2 : 5 : 6-DIBENZANTHRACENE.

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THE sarcomata induced artificially in the fowl are of great interest in view of the important problem of the relation between the mammalian sarcomata and the filterable fowl tumours of the Rous type. Successful induction of tumours in the fowl has been reported by Burrows (1933), Peacock (1933), McIntosh (1933) and Haddow (1934). Of these workers, Burrows, Peacock and Haddow used injections of 1 : 2 : 5 : 6-dibenzanthracene dissolved in lard as the inducing agent, whilst Peacock and McIntosh describe tumours produced by means of injections of tar. The tumours were all difficult to propagate by transplantation. Of Peacock's tumours two were grafted successfully through six generations, but failed in the seventh. None yielded an infective filtrate. McIntosh reports the propagation of two tar-induced tumours by means of Berkfeld filtrates, and Haddow obtained an infective filtrate from a tumour induced by 1 : 2 : 5 : 6-dibenzanthracene in lard.

When the experiments to be described in this paper were commenced, the carcinogenic activity of 1 : 2 : 5 : 6-dibenzanthracene had been demonstrated only by the effects of its application in conjunction with a solvent. For intramuscular injection the solvent generally used was lard. It was thought desirable to make quite certain that the carcinogenic activity of the hydrocarbon was not in any way dependent on the presence of the simultaneously administered fat solvent. The fact that Peacock reports the appearance of three tumours as the result, apparently, of injections of lard alone heightens the necessity for evidence on this point.

In a preliminary test 12 rats were given an injection in the groin of a suspension of 1 p.c. of 1 : 2 : 5 : 6-dibenzanthracene in physiological saline containing 6 p.c. gum acacia. Six of these rats received 2·5 mg. and 6, 5 mg. dibenzanthracene. An epidemic was responsible for the death of all but 3 rats within 3 months, but of these, 2 (2·5 and 5·0 mg.) developed tumours at the site of injection in less than 6 months, and the third (5·0 mg.) after 7 months. The tumours grew rapidly to a large size, and were identified histologically as spindle-cell sarcomata. Grafts from one of them grew well in 5 out of 6 rats, and in section appeared similar to the primary tumour. On further transplantation a high percentage of takes was obtained, but these all ultimately regressed, so that no histological evidence of their nature is available.

The discovery by Berenblum (1932) of a method for preparing a colloidal solution of 1 : 2 : 5 : 6-dibenzanthracene in water made it possible to examine the results of its administration in this more satisfactory form. A similar colloidal solution prepared at about the same time, independently and by a different method, by Boyland (1932), has been reported to be effective in inducing sarcomata in rats (1933).

EXPERIMENTAL.

The Production of Tumours with Colloidal Dibenzanthracene.

The dibenzanthracene solution was prepared by the method described by Berenblum, and contained rather less than 10 mg. per 100 c.c. of water.* Eighteen young Plymouth Rock cockerels were given weekly injections into the left breast muscle of 2 c.c. of this solution. Six birds died within the first 12 weeks, after which time the injections were discontinued. The remaining birds had each received, therefore, about 1·8 mg. dibenzanthracene. They were examined at intervals of 2 weeks, and the results were as follows :

(A) Three of the 12 survivors showed no sign of any reaction to the injected substance up to the time of their deaths, 28 weeks, 58 weeks and 80 weeks respectively after the first injection.

(B) One bird developed a swelling after 13 weeks, which grew progressively, but proved to be of an inflammatory nature when examined histologically after the animal had died (38 weeks).

* Owing to some precipitation during dialysis the final solution always contained less than 10 mg. per 100 c.c. Subsequent estimation by a spectrographic method showed that the amount present lay between 6 and 8 mg. per 100 c.c. in different samples. The concentration of the solution used is therefore assumed to have been about 0·007 p.c.

(c) In one bird there was no apparent reaction at the site of injection, but about 5 cm. away, near the lower margin of the left side of the sternum, a swelling was noticed at the 15th week. This reached a diameter of about 2 cm., then diminished in size, and at post-mortem proved to be a tumour of the dimensions $3 \times 0{\cdot}5 \times 0{\cdot}5$ cm.

(d) The remaining 7 birds all developed tumours at the site of injection. With one exception these tumours increased in size until the deaths of the animals, although the rate of growth varied considerably. It is interesting to note that those tumours which appeared first, *i. e.* 5 palpable at the 13th week after the first injection, all grew very slowly for several months, while the 2 which appeared much later (after 29 and 37 weeks respectively) grew rapidly, reaching a diameter of about 4 cm. in 3 weeks. In one of the fowls a swelling which was noticed at the 13th week had attained a diameter of 2–3 cm. by the 29th week. It then diminished in size, until by the 37th week it was scarcely palpable. Between the 40th and 48th weeks a phase of rapid growth set in, and at the end of this time it was 4–5 cm. in diameter. At death, after 62 weeks, the dimensions of this tumour were $11 \times 4 \times 4$ cm.

The tumours could all be classified as spindle-cell sarcomata, though there was considerable variation in the amount of fibrous tissue and of necrotic material present, in the frequency of mitosis and in the degree of lymphocytic reaction in the individual specimens. All showed invasion of muscle, and in the sections of some of them a number of tumour giant-cells could be seen.

Five of the birds which developed tumours appeared to be free from metastases, but in 3 birds secondary deposits were found in the heart, and 1 of these had, in addition, a large secondary growth, $10 \times 7 \times 6$ cm. in size, in the liver. The secondary growths were similar in character to the corresponding primary tumours, but in each case they were more active and less fibrous.

Attempts were made to propagate 5 of the tumours by transplantation into other birds. Quantities of 0·5 c.c. of tumour mince were injected into the left breast muscle of young Plymouth Rock fowls, but circumstances unfortunately limited the total number of birds which were used for this purpose to 23 only.

In the case of three of these tumours no positive result was obtained. Six birds which had been inoculated with one tumour showed no signs of the grafts having taken after 10 weeks. One of these birds became sickly and was killed 14 weeks after inoculation. It was found to have, at the site of inoculation, a nodule about 1 cm. in diameter, which histological examination showed to be a tumour similar to the original tumour. In the remaining 5 birds of this group no sign of the inoculation was to be seen when post-mortem examinations were made after 12 months.

Transplantation was successful in one other case. A minced primary tumour was injected into 3 birds, and minced tumour tissue taken from a very active secondary growth in the heart from the same animal was injected into a further 3 birds. In one of the second group of 3 a tumour developed at the site of inoculation. After 9 weeks the fowl died, and, in addition to the tumour in the breast, which measured $4 \times 3 \times 2$ cm., there was a secondary deposit in the liver.

The Effect of Kieselguhr on Tumour Formation with Colloidal Dibenzanthracene.

The above experiments demonstrated that sarcomatous tumours can readily be induced by intramuscular injections of dibenzanthracene in the absence of any solvent other than water. The possibility still remained, however, that the fatty medium used by previous workers influenced the induction of tumours either (*a*) by presenting the dibenzanthracene in a more suitable physical state, or (*b*) by virtue of the local inflammatory reaction which it might produce. It was decided to test the latter possibility by injecting, in conjunction with colloidal dibenzanthracene, a substance which is chemically inert yet capable of producing a well-marked inflammatory reaction. Kieselguhr was chosen for the purpose.

Three groups of 12 fowls were used. Group A received injections of a suspension of 0·5 p.c. kieselguhr in water; Group B received injections of 0·5 p.c. kieselguhr suspended in the aqueous colloidal solution of dibenzanthracene; while in Group C the dibenzanthracene solution alone was injected. It was thought that any influence which kieselguhr might have on tumour formation would be more readily demonstrable if smaller quantities of dibenzanthracene were used than were found adequate in the previous experiment. Eight fortnightly injections of 1 c.c. of the above-mentioned suspensions were therefore given, the total amount of dibenzanthracene received by each bird in Groups B and C being about 0·6 mg.

Of the 36 birds, 29 survived more than 12 months, while 21 were still alive after 18 months. These were then killed and examined. Only 1 animal developed a tumour. It was palpable at the site of injection after 8 months in a bird of Group B (kieselguhr and dibenzanthracene), and grew rapidly in the following 3 weeks, but thereafter more slowly. The fowl died three months after the first appearance of the growth, which at post-mortem measured $12 \times 5 \times 5$ cm. Extensive secondary deposits were present in both lungs. Histologically the tumour appeared to be an actively growing spindle-cell sarcoma with a large central area of necrosis. The tumour was grafted into the nine young birds without success.

In many of the birds of Groups A and B small yellowish-white nodules were found in the muscle at the site of injection at post-mortem. Those in Group B were on the average definitely larger than those in Group A, but histologically the appearance in each case was an inflammatory reaction, with kieselguhr particles distributed throughout the lesion.

The Disappearance of Dibenzanthracene at the Site of Injection.

An attempt was made to discover whether any dibenzanthracene was still present at the site of injection in 7 of the birds from Groups B and C, when these were killed at the termination of the above experiment. A wide area of muscle around the site of injection was excised, minced and desiccated *in vacuo* over phosphorus pentoxide. When thoroughly dry the material was ground to a fine powder, and extracted with benzene for 6 hours in a Soxhlet apparatus. Each extract was then examined in ultra-violet light for the

fluorescent spectral bands which are characteristic for 1 : 2 : 5 : 6-dibenzanthracene. No evidence of such bands was obtained. Tests of the sensitivity of the method indicated that the amount of dibenzanthracene present in each extract could not have been more than 0·02 mg.

In order to show that the extraction procedure was adequate, 2 control birds were given intramuscular injections of 5 c.c. colloidal dibenzanthracene (about 0·35 mg.) and killed 1 hour afterwards. The muscle was excised, desiccated and extracted as described above, and the extracts, when examined spectrographically, showed the expected bands with an intensity corresponding to 0·2–0·25 mg. of dibenzanthracene.

Since each of the 7 birds initially received 0·6 mg. of dibenzanthracene, while less than 0·02 mg. was present in the breast muscle at the end of the experiment, most of the dibenzanthracene must have disappeared from the site of injection during the course of the experiment.

DISCUSSION.

The experiments described above confirm the view that the carcinogenic activity of 1 : 2 : 5 : 6-dibenzanthracene is not dependent on the presence of any other substance foreign to the tissues. They demonstrate, moreover, that repeated injections of a colloidal solution of dibenzanthracene is a satisfactory and convenient method of inducing sarcomata in the fowl.

A consideration of the periods of time which elapsed before the tumours became palpable is of some interest. Of the 7 tumours which developed at the site of injection, 5 became palpable after 13 weeks, 1 after 29 weeks and 1 after 37 weeks. The 5 which appeared at the 13th week all grew very slowly for several months before assuming a relatively rapid rate of growth, while the 2 which were discovered late grew rapidly from the time at which they were first detected. In assessing the significance of these results, two possible explanations must be borne in mind. Those swellings which developed at an early date may not have become malignant until the time of onset of rapid growth some months later. Alternatively, the 2 tumours which were first observed after a long latent period may have originated very early, but may have remained too small or too deeply situated to be detected by palpation.

In view of the large yield of tumours following the administration of 1·8 mg. of dibenzanthracene in the first experiment described above, it was somewhat surprising to find that in the subsequent experiment (Group C) 0·6 mg. of dibenzanthracene failed to produce a single tumour. Actually, in the latter group not only was the total amount of dibenzanthracene smaller, but the individual injections were also smaller (1 c.c. instead of 2 c.c.) and were spread out over a longer period (fortnightly injections instead of once a week). That this may have accounted for the striking difference in the yield of tumours is made more probable by the subsequent observation that the animal is capable of either destroying or removing the dibenzanthracene from the site of injection. This disappearance of dibenzanthracene after local injections has also been described by Chalmers (1934). It is conceivable that when 1 c.c. of dibenzanthracene is injected once a fortnight into the breast muscle of a fowl the animal is capable of removing it before any carcinogenic effect is

produced, whereas when 2 c.c. are injected weekly, sufficient dibenzanthracene remains to initiate the development of a tumour.

The fact that one tumour was produced by the action of kieselguhr together with 0·6 mg. of dibenzanthracene (Group B) while none appeared with 0·6 mg. of dibenzanthracene alone (Group C) cannot be considered significant. Thus no evidence was obtained to support the view that a local inflammatory lesion facilitates the initiation of tumour formation. Yet Lacassagne (1933) has recently shown that an inflammatory lesion produced by kieselguhr occasionally developed into a tumour after subjecting the lesion to X-rays. We do not know, however, whether the mechanism of carcinogenesis with X-rays is the same as that with a specific chemical agent like dibenzanthracene.

SUMMARY.

1. Repeated injections of a 0·007 p.c. colloidal solution of dibenzanthracene in water into the breast muscle of the fowl led to the production of spindle-cell sarcomata in 8 of 12 birds which survived more than 12 weeks. The total amount of dibenzanthracene injected was about 1·8 mg. Secondary deposits were found in 3 birds.

2. Five of these tumours were transplanted into other birds, and of these 2 grew successfully. In one of these inoculated birds metastases were present in the lungs.

3. The administration of kieselguhr together with colloidal dibenzanthracene did not appear to influence significantly the development of tumours.

4. Eighteen months after intramuscular injections of 0·6 mg. of dibenzanthracene, none of the substance could be detected in the muscle about the site of injection.

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THE IMMUNOLOGICAL RELATIONSHIPS OF PSEUDORABIES (INFECTIOUS BULBAR PARALYSIS, MAD ITCH).

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PSEUDORABIES was first recognized as a distinct disease of certain domestic animals (ox, dog, cat) by Aujeszky (1902). He also established the ætiological agent as an ultramicroscopic virus and differentiated it from true rabies on the basis of certain clinical features, the non-transmission of the disease by the bite of an infected animal, and the difference in the pathological picture, the outstanding features of which were perhaps the absence of obvious pathological change and of the classical Negri bodies. The disease was then observed among many different animals (cattle, sheep, swine, fox, etc.), and has also been given the name of " infectious bulbar paralysis ". Recently Shope (1931) demonstrated that a disease of cattle known in the U.S.A. as " mad itch " is caused by the same virus. However, it was still maintained by certain investigators (Nicolić, 1932) that the virus of pseudorabies was a modified form of rabies, and it was not until Hurst (1933) investigated the pathology of the disease in various animals that the virus was established on a more definite and distinct footing. He showed that the characteristic cellular change associated with pseudorabies was not a cytoplasmic " inclusion ", but rather an acidophilic, intranuclear inclusion of the same type as that which occurs with herpes, Virus III, yellow fever and others. He also demonstrated that the virus had affinities for cells derived from all the embryonic layers. Traub (1933) subsequently observed the same intranuclear inclusions in all types of cells of minced testis in in-vitro cultures of pseudorabies. In its pluricellular affinities, as well as in the type of intranuclear inclusion, it closely resembles herpes simplex, and it would indeed be most difficult to differentiate the histopathological picture produced by the two viruses. A virus called the B virus, recently isolated from a human case of myelitis associated with visceral necrosis which followed a monkey-bite (Sabin and Wright, 1934), was shown to be even more closely related to pseudorabies both as regards the histopathology, host susceptibility and clinical features (Sabin, 1934*a*). The cardinal sign of pseudorabies, which is pruritus, leading to the licking and biting of the zone of inoculation, was observed in about 30 p.c. of rabbits injected with the B Virus, but what is more important is that a certain number of animals which were rendered immune to B Virus proved also immune to a minimal though constantly infective dose of pseudorabies ; furthermore, certain anti-B

Virus sera were capable of protecting against a similar minimal amount of pseudorabies. Quantitative studies showed that although these two viruses could readily be differentiated by serological methods, there was nevertheless a slight immunological relationship. Since a similar partial immunological relationship was shown to exist between the B Virus and herpes simplex, the primary purpose of the present investigation was to determine the relationship, if any, between the latter virus and pseudorabies.

EXPERIMENTAL.

The pseudorabies virus used in the present investigation was the Aujeszky strain. The antipseudorabies serum was obtained from immune swine, and was kindly supplied by Dr. R. E. Shope, of the Rockefeller Institute, Princetown, N.J. Two strains of herpes virus were employed : one was the Perdrau EL_1 rabbit strain, and the other Bedson's dermotropic guinea-pig strain. The anti-herpes sera were derived either from rabbits or guinea-pigs, as indicated, and before use were all shown to contain herpes anti-viral bodies. The active immunity tests were unilateral of necessity, since sublethal amounts of pseudorabies do not immunize rabbits or guinea-pigs, and the immunization of guinea-pigs by other methods is not readily accomplished.

Reaction of Herpes-Convalescent Rabbits to Infection with Pseudorabies by the Intracutaneous Route.

The rabbit is the most susceptible animal for pseudorabies, the minimal infective dose invariably leading to the characteristic syndrome and death. Nine herpes-convalescent rabbits were tested by the intracutaneous injection of an amount of pseudorabies which was within the minimal range of effectiveness and was less than 10 M.I.D. All of these rabbits had recovered from multiple, typical herpetic (Perdrau, EL_1 strain) skin lesions, and three of them had additional inoculations of virus. One of these rabbits (No. 112) had also recovered from Virus III skin lesions. The intervals between the last inoculation of herpes and the test with pseudorabies varied from 21 to 70 days. No special attempt was made to inject in a previously uninoculated area. Two vaccinia-convalescent and five normal rabbits served as controls. The results are shown in Table I. With the exception of No. 167, none of the herpes- or the vaccinia-convalescent rabbits reacted differently from the normal ones. Rabbit 167 presented an especially interesting case. Within less than 24 hours after the injection of pseudorabies and 22 days after the inoculation of herpes it developed spastic paralysis and generalized tremors without any evidence of local lesions or irritation at the site of the test inoculation. There was no question that these signs were due to a delayed herpetic myelo-encephalitis ; four similar cases of delayed herpetic involvement of the central nervous system had been observed in this laboratory recently. These rabbits lived for several days following the development of signs, and histologically showed destructive lesions in the white matter in addition to those in the grey. What is of interest here, however, is the apparent modification of the course of pseudorabies when superimposed on such a process. Judging from the time at which

this rabbit began biting, the virus apparently reached the spinal ganglia with the usual rapidity, but whereas all the other rabbits died within less than 24 hours after the onset of biting, this rabbit continued to bite for two days, first the inoculated zone, and then the corresponding area of the uninoculated side. On the third day after the onset of biting the rabbit was still alive, though prostrate. It was sacrificed, and histological examination of the spinal cord revealed, in addition to other changes, destructive lesions in the white matter.

TABLE I.—*Reaction of Herpes-Convalescent Rabbits to Infection with Pseudorabies by the Intracutaneous Route.*

History.	Rabbit No.	Amount of virus.	Result.
Normal controls	171	0·1 c.c., 1 : 1 : 10,000	No lesion; survived.
	198	0·1 c.c., 1 : 1000	S_2, Bit. 4, D. 5.*
	178	0·2 c.c., 1 : 1000	S_1, Bit. 3, D. 4.
	179	„ „	S_2, Bit. 3, D. 4.
	172	0·1 c.c., 1 : 100	„ „
Vaccinia-convalescent	180	0·2 c.c., 1 : 1000	„ „
Four series of intracutaneous inoculations of herpes virus at 5-day intervals; last one 30 days before test	187	„ „	„ „
Three series of intracutaneous and subcutaneous herpes inoculations at 10-day intervals; last one 35 days before test	133	„ „	S_1, Bit. 3, D. 4.
Had multiple Virus III skin lesions 68 days before, followed by three series of intracutaneous herpes inoculations at 10-day intervals; last one 35 days before test	112	„ „	„ „
Multiple herpetic skin lesions 36 days before test	152	„ „	S_2, Bit. 3, D. 4.
Multiple herpetic skin lesions 25 days before test	166	„ „	S_1, Bit. 3, D. 4.
Multiple herpetic skin lesions 21 days before test	167	„ „	Signs of myelencephalitis first day (delayed herpetic); skin lesion did not develop; bit site of inoculation 3rd day, remained alive on 4th day biting inoculated zone as well as corresponding region of the opposite uninoculated side. Prostrate but still alive on 5th day, when he was sacrificed.
Multiple herpetic skin lesions 70 days before test	183	0·1 c.c., 1 : 1000	S_2, Bit. 3, D. 4.
Multiple herpetic skin lesions 35 days before test	215	„ „	„ „
Same as 215	216	„ „	„ „
Vaccinia convalescent	231	„ „	„ „
Normal control	236	„ „	„ „

* S_2, Bit. 4, D. 5: Skin lesion 2nd day, biting of inoculated zone 4th day, and death 5th day after inoculation.

TABLE II.—*Reaction of Herpes-Treated Guinea-pigs to Infection with Pseudorabies.*

Group.	Preliminary treatment.	Guinea-pig No.	Date.	Amount of pseudorabies subcutaneously.	Result.
Herpes-convalescent; Bedson's dermal strain	16.vi.34 injected into pads, scarified on skin, and 1 c.c. intramuscularly	86	6.vii.34	0·5 c.c., 1 : 100	D.<69.
		87	„	„ „	D. 76.
		88	„	„ „	Survived.
			31.viii.34	1·0 c.c., „	D. 72.
	21.vi.34 skin scarified, pads injected, 0·75 c.c. subcutaneously	89	6.vii.34	0·5 c.c., 1 : 100	Survived.
			31.viii.34	1·0 c.c., „	D. 88.
		90	6.vii.34	„ „	D.<69.
Normal control	None	72	6.vii.34	1 c.c., 1 : 1000	D. 80.
		62	„	„ „	D. 96.
		91	„	0·5 c.c., 1 : 100	D.<69.
		92	„	„ „	D. 70.
		93	„	„ „	D. 76.
		94	„	„ „	Survived.
		43	„	1 c.c., „	D. 80.
		74	„	„ „	D. 80.
Herpes; Perdrau EL_1 strain	20.vi.34 to 7.viii.34 were given four series of subcutaneous inoculations of herpetic brain	100	31.viii.34	0·5 c.c., 1 : 100	D. 120.
		101	„	„ „	D. 72.
		102*	„	„ „	Survived.
		103	„	„ „	D. 72.
		104*	„	„ „	Survived.
		105	„	„ „	D. 88.
		106	„	„ „	D. 69.
		107	„	„ „	D. 69.
		108	„	„ „	D. 69.
Vaccinia and tissue control	1 c.c. of neurotesticular virus (rabbit) subcutaneously on 1.viii.34 and 7.viii.34	109	„	„ „	D. 70.
		110	„	„ „	D. 71.
		111	„	„ „	D. 69.
		112	„	„ „	D. 69.
		113	„	„ „	D. 88.
Normal control	None	95	„	„ „	D. 70.
		96	„	„ „	D. 88.
		97	„	„ „	D. 90.
		98	„	„ „	D. 130.
		99	„	1 c.c., 1 : 1000	D. 88.

* Guinea-pigs 102 and 104 succumbed when tested subsequently with 1 c.c. of a 1 : 100 dilution of virus.

D.<69 = Found dead with evidence of biting 69 hours after inoculation.

Since a similar delay in the progress of pseudorabies was never observed among any other animal of many rabbits and guinea-pigs injected with this strain, it is not improbable that the lesions already existing in the spinal cord of this rabbit hindered in some way the rapid ascent of the virus to the medulla. As far as any immunological relationship between herpes and pseudorabies is concerned, none was demonstrable in the tests on rabbits.

Reaction of Herpes-Convalescent Guinea-pigs to Infection with Pseudorabies.

Although in the tests on rabbits there was no evidence whatever of any immunological relationship between herpes and pseudorabies, it was appreciated, nevertheless, that partial relationships may become apparent only in

TABLE III.—*Effect of Antiherpes Sera on Pseudorabies in Guinea-pigs.*

Serum.	Amount (c.c.).	Dilution of pseudorabies.	Amount (c.c.).	Guinea-pig No.	Result.	Date.
None; saline control	1·0	1 : 10	1·0	11	D. 70	
	1·0	1 : 100	1·0	12	D. 70	
	1·0	1 : 1000	1·0	13	D.<92*	29.v.34.
Anti-herpes — W.S. pooled hyperimmune rabbit serum	1·0	1 : 10	1·0	19	D.168†	
	1·0	1 : 100	1·0	20	Survived	
	1·0	1 : 1000	1·0	21	„	
None; saline control	1·0	1 : 100	1·0	27	D.<96	
	1·0	1 : 1000	1·0	26	D. 73	
	1·0	1 : 10,000	1·0	25	Survived	
Normal rabbit 1	1·0	1 : 10	1·0	29	D. 72	
	1·0	1 : 100	1·0	28	D.<70	
„ „ 2	1·0	1 : 100	1·0	31	D.<70	
	1·0	1 : 1000	1·0	30	D.<84	
„ „ 3	1·0	1 : 100	1·0	33	D.<70	
	1·0	1 : 1000	1·0	32	Survived	5.vi.34.
R. 73—Anti-herpes; hyperimmune rabbit	1·0	1 : 10	1·0	36	D.<70	
	1·0	1 : 100	1·0	35	Survived	
	1·0	1 : 1000	1·0	37	D. 74	
R.5—Anti-herpes; hyperimmune rabbit	1·0	1 : 10	1·0	39	D. 72	
	1·0	1 : 100	1·0	38	Survived	
	1·0	1 : 1000	1·0	37	„	
R.105 — Anti-herpes; convalescent rabbit	1·0	1 : 10	1·0	42	D.<70	
	1·0	1 : 100	1·0	41	D. 70	
	1·0	1 : 1000	1·0	40	Survived	
None; saline control	1·0	1 : 1000	1·0	45	D.<70	
	1·0	1 : 1000	1·0	44	Survived	
	1·0	1 : 10,000	1·0	43	„	
R.111 — Anti-herpes convalescent	1·0	1 : 10	1·0	58	D. 79	12.vi.34.
	1·0	1 : 100	1·0	57	D.<94	
Anti-virus III	1·0	1 : 100	1·0	60	D.<70	
	1·0	1 : 1000	1·0	59	Survived	
P.G.P.—Guinea-pig anti-herpes	0·5	1 : 100	0·5	114	Survived	
	1·0	1 : 100	0·5	115	„	
	1·0	1 : 100	1·0	116	„	
	1·0	1 : 10	0·5	117	D. 90	
H.G. anti-herpes; hyperimmune guinea-pig	0·5	1 : 100	0·5	118	Survived	
	1·0	1 : 100	1·0	119	D. 72	
H_4A — Anti-herpes; hyperimmune guinea-pig	0·5	1 : 100	0·5	120	Survived	
	1·0	1 : 100	0·5	121	„	1.ix.34.
	0·5	1 : 100	1·0	122	„	
	0·8	1 : 10	0·5	123	D. 90	
Normal guinea-pig	0·5	1 : 100	0·5	124	Survived	
	1·0	1 : 100	1·0	125	„	
	1·0	1 : 10	0·5	126	D. 90	
Anti-vaccinial rabbit serum	0·5	1 : 100	0·5	127	Survived	
	1·0	1 : 100	1·0	128	„	

animals for which the virus is least virulent. For this reason it was decided to repeat the previous experiment on guinea-pigs. Five guinea-pigs were inoculated with Bedson's dermal guinea-pig strain, and nine others received four series of subcutaneous injections of 1 c.c. of a 20 p.c. rabbit herpetic brain (Perdrau, EL_1) suspensions at 6-day intervals. The minimal test dose of pseudorabies was 0·5 c.c. of a 1 : 100 (1 p.c.) rabbit brain suspension injected subcutaneously. Although one-fifth of that amount, *i. e.* 1 c.c. of 1 : 1000 suspension, is fatal for over 50 p.c. of guinea-pigs, the larger dose was selected because it was found to be almost constantly fatal in a preceding investigation. Eleven normal guinea-pigs and 5 guinea-pigs treated with a rabbit strain of neuro-testicular vaccinia served as controls, all being injected with the same pseudorabies suspension used for testing the herpes-treated guinea-pigs. The results recorded in Table II show that the 5 vaccinia-treated guinea-pigs and 10 of the 11 normal ones died of pseudorabies, while 4 of the 14 herpes-treated guinea-pigs, or almost a third, were resistant to this dose. The 4 resistant guinea-pigs succumbed typically when re-tested with a larger dose. Although B virus-immune guinea-pigs were found to be relatively more resistant to pseudorabies (Sabin, 1934*a*) than the herpes-convalescent guinea-pigs in the present series, these results, nevertheless, suggest a possible partial relationship which warranted further serological investigation.

Effect of Herpes-Convalescent and Hyperimmune Sera on Pseudorabies in Guinea-pigs.

A number of anti-herpes sera which had been titrated in a previous investigation (Sabin, 1934*a*) were tested against varying amounts of rabbit pseudorabies brain suspension. The indicated amounts of serum were mixed with virus and left at room temperature for 2 hours and in the cold room overnight; the total amount of the mixture was then injected subcutaneously in a guinea-pig. Since virus suspension conceivably may vary in potency, each test was accompanied by a titration of the virus used, and in the evaluation of the results presented in Table III it is essential to compare the effects of the serum-virus mixtures with the controls of the same day. Three hyperimmune and two convalescent herpes rabbit sera which were prepared with the Perdrau EL_1 strain and three hyperimmune guinea-pig sera were tested. Three normal rabbit sera, a normal guinea-pig serum, an anti-Virus III serum and an anti-vaccinial serum served as controls. Two hyperimmune rabbit sera, "W.S." and R5, definitely protected against small but constantly infective amounts

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After an empirical incubation for 2 hours at room temperature and overnight in the cold room, the total amount of the mixtures was injected subcutaneously.

* D.<92 = Found dead 92 hours after inoculation with signs of pseudorabies having been observed the day before. When the "less" sign is not used it signifies that the animal died during the day when the exact number of hours following inoculation could be determined.

† Guinea-pig showed no evidence of biting; there was only slight salivation; death was probably due to a modified pseudorabies.

Sera H.G. and H_4a were prepared with the Bedson dermal strain of herpes. The sera were kindly supplied by Dr. M. H. Salaman, of Prof. Bedson's laboratory.

All the other anti-herpes sera were prepared with the Perdrau EL_1 strain, and were shown to be potent by titration in rabbits.

of pseudorabies; it is interesting to note that these two sera were also the ones which protected against minimal quantities of B Virus (Sabin, 1934*a*). The herpes-convalescent sera and the antivirus III serum were apparently without effect. The tests with the guinea-pig antisera are inconclusive because the virus suspension was apparently of low potency; they were no better, however, than the normal guinea-pig serum. In general the results of this test are similar to those obtained in the investigation on the relationship of the B Virus to herpes simplex. It may be interesting to add that the hyper-immune herpes sera which "neutralized" small amounts of pseudorabies and B Virus had no effect whatever on Virus III, which may perhaps be considered as a point in favour of the specificity of their action.

Effect of Anti-pseudorabies Serum on Herpes.

In view of the preceding findings, it was interesting to determine whether an anti-pseudorabies serum would have any effect on small doses of herpes. A potent immune swine serum was tested against a herpetic testicular suspension (Perdrau EL_1), and a normal swine serum was used for control. The serum-virus mixtures were injected intracutaneously. Although both the normal and immune swine sera gave rise to early inflammatory reactions, there was no difficulty in determining the development of the herpetic lesion, because of the characteristic vesicular appearance of the latter. The results recorded in Table IV show that the anti-pseudorabies serum had no effect whatever. A similar test was carried out with a monkey convalescent anti-pseudorabies serum, but the results were inconclusive because the serum of this monkey, which was obtained before infection with pseudorabies and used as a control, neutralized herpes. The negative results obtained with the immune swine serum are important in ruling out the remote possibility that the acidophilic intranuclear inclusion observed with pseudorabies may be due to a contamination with herpes.

TABLE IV.—*Effect of Anti-pseudorabies Serum on Herpes.*

Serum.	Concentration of herpes.			
	1 : 20.	1 : 100.	1 : 1000.	1 : 10,000.
Saline control	n.t.	++ ves.*	+++ ves.	—
Anti-pseudorabies swine serum	+++ ves.	+++ ves.	+++ ves.	n.t.
Normal swine serum control	+++ ves.	+++ ves.	+++ ves.	n.t.
Anti-herpes serum control	—	—	—	n.t.

0·2 c.c. of serum-virus mixtures injected intracutaneously on a rabbit's back.
n.t. = not tested. * ves. = vesicles.

Effect of Anti-rabic Serum on Pseudorabies.

Although the assumption that pseudorabies is a modified form of rabies virus (Nicolić, 1932) became untenable with the demonstration of the distinct pseudorabies histopathology, it was nevertheless interesting to test the effect of anti-rabic serum against small doses of pseudorabies. The anti-rabic serum was kindly supplied by Dr. Hurst, whose tests showed that it neutralized rabies by the cerebral route. Mixtures consisting of 1 c.c. of this serum

with 1 c.c. of 10 p.c. and 1 c.c. of 1 p.c. rabbit pseudorabies brain suspension were injected subcutaneously into guinea-pigs. There was no evidence of any modification of the course of the disease, and the guinea-pigs succumbed in the usual time and manner.

DISCUSSION.

The purpose of the present investigation was to determine the immunological identity of the pseudorabies virus. The contention that it was a modified form of rabies became untenable with the demonstration of the acidophilic intranuclear inclusion body that is associated with it, and of its pluricellular affinities (Hurst, 1933). Recently it was shown that the B Virus resembled pseudorabies in many respects, particularly with regard to certain clinical manifestations in experimental animals, to histopathology, and host susceptibility. Although these two viruses could be differentiated immunologically and serologically, quantitative tests suggested the existence of a partial immunological relationship between them (Sabin, 1934*a*). Since the B Virus was shown to bear a similar relationship to herpes, it appeared possible that these three viruses, which have so many properties in common, may also be immunologically related. The results of the present investigation indicate that a certain number of herpes-immune guinea-pigs may resist small but definitely infective amounts of pseudorabies, and that certain hyperimmune anti-herpes sera may protect guinea-pigs against similar small doses of pseudorabies virus. It is fully realized that the present data only suggest a relationship between herpes and pseudorabies—they do not prove it. Among the ordinary bacteria group relationships may readily be established by the demonstration of a common antigen. Whether or not relationships among filterable viruses may have a similar basis is not known, but it is not inconceivable that other mechanisms involving the parasitized cell may be involved.

If one were to classify filterable viruses, there would appear to be sufficient justification to put pseudorabies, the B Virus and herpes in a single group. Their generic relationship may perhaps be based on the following properties, which, taken together, are not possessed by any other virus: (*a*) They are pantropic, *i. e.* they have affinities for cells derived from all three embryonic layers; (*b*) they are neuro-invasive, *i. e.* they are capable of invading the central nervous system from a peripheral focus; (*c*) they give rise to a similar intranuclear inclusion body; (*d*) they have a similar, though not identical, range of susceptible hosts; and (*e*) they appear to have a partial immunological relationship. Their natural hosts, however, would seem to be distinct; thus herpes is found in man, pseudorabies among cattle and certain domestic and wild animals, and from what is known of the B Virus its natural host is probably the *rhesus* monkey (Sabin, 1934*b*). Yet the rabbit is the most susceptible experimental animal for all. The differences in the course and type of disease produced in the rabbit by these three viruses appear to be more variations of degree than of kind. Thus a rabbit injected intracutaneously with any one of these viruses shows similar pathological changes in the spinal ganglia and in the cord at the level of inoculation; yet with pseudorabies the spinal ganglia are reached rapidly and almost all the animals show clinical

signs of their involvement, *i. e.* pruritus with licking and biting of the corresponding zone; with the B Virus the ganglia are reached more slowly, and in only about 30 p.c. of the animals is there sufficient change to give rise to clinical signs, whereas with herpes these clinical signs, though extremely rare, are not unknown. Again, whereas with the B Virus there is in all rabbits sufficient change at the portal of entry in the spinal cord to give rise to paralysis of the affected muscles, with herpes this manifestation is less common, most strains giving rise to an encephalitis. Thus, while attacking the same structures, they may differ in the degree in which they can damage particular types of cells.

SUMMARY.

1. Pseudorabies bears no immunological relationship to rabies.

2. While possessing many properties in common with the virus of herpes simplex, it is easily differentiated from it by active and passive immunity tests.

3. Certain hyperimmune anti-herpes sera protected guinea-pigs from minimal but constantly infective doses of pseudorabies.

4. Four of fourteen herpes-immunized guinea-pigs resisted a small but definitely infective dose of pseudorabies.

5. A potent anti-pseudorabies serum had no effect on herpes.

6. A partial immunological relationship between pseudorabies and herpes is considered possible.

7. A generic relationship is suggested for pseudorabies, the B Virus and herpes simplex.

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THE FLOCCULABLE SUBSTANCE OF VACCINIA: THE EFFECT ON ITS ANTIGENIC PROPERTIES OF ADSORPTION ON COLLODION PARTICLES.

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INTRODUCTION.

ALTHOUGH the present method of prophylactic inoculation against smallpox is undoubtedly effective, it falls short of the ideal in that it is not devoid of risk. Admittedly the incidence of post-vaccinial encephalitis is extremely low, but even so the chance of producing such a serious sequela constitutes a definite drawback to the method, and makes the search for a superior one highly desirable. In the past a number of attempts have been made to obtain an effective vaccine of killed vaccinia virus. Such agents as heat, formol and phenol have been used for this end, but only with a very small measure of success; with none of these preparations has it been possible to produce a reasonable degree of immunity in experimental animals. Recent work has suggested the possibility of a new approach to this problem. In his investigations of the antigenic structure of vaccinia virus—made possible by the perfection of the vaccinia-variola flocculation reaction by Burgess, Craigie and Tulloch (1929)—Craigie (1932*a*) has shown that Seitz filtrates of suspensions of lapine (rabbit dermal strains of vaccinia), although non-infective, contained a substance which flocculated specifically with anti-vaccinia serum. He has also shown that an anti-vaccinia serum absorbed with such a flocculable filtrate loses not only its power of flocculating the filtrate, but also of agglutinating the washed elementary bodies; a similar result was obtained when absorption was done with washed elementary bodies. From this Craigie has concluded that the Seitz filtrate contains a specific product of vaccinia virus. Wilson Smith (1932) obtained extracts from a rabbit testicular strain of vaccinia by a method comprising autolysis at 37° C. and subsequent boiling. This extract, like Craigie's filtrate, gave specific flocculation, and was, of course, non-infective. These derivatives differed not only in their source of origin and method of preparation, but also, apparently, in some of their properties. Craigie, for instance, found that the filtrate lost its specific flocculating power when heated at 55° C., just as crude vaccinia suspensions had been found to do (Craigie and Tulloch, 1931). Wilson Smith's extract withstood repeated boiling. Apart from this, however, these two preparations showed similarities. Neither evoked antibody when injected into rabbits, though Craigie's filtrate gave rise to an increase in antibody titre when administered to rabbits

previously infected with vaccinia virus (Tulloch, 1932). Both produced an allergic reaction in previously vaccinated humans, but not regularly in rabbits (Wilson Smith, 1932 ; Craigie and Wishart, 1933). It would appear from this evidence that vaccinia virus probably possesses an antigenic constituent similar in character to the specific soluble substances or haptenes of bacteria, and that the Seitz filtrate of a vaccinia suspension contains this substance, some of it free, some possibly still bound to fragments of elementary bodies. The composition of the Seitz filtrate will be discussed later, but apparently one possesses in such a preparation the antigenic components of vaccinia virus, unaltered by heat or chemicals and devoid of infectivity, but unfortunately also devoid or almost completely devoid of antigenic power in the strict sense of the term. Immunological research of late years has provided abundant proof that the half-antigens or haptenes can be restored to full antigenic activity if they are linked chemically or physically to large molecules, such as the protein molecule. The work of Zozaya (1932) has shown that in this *rôle* the protein molecule can be replaced by relatively large inert colloidal particles, such as collodion or charcoal, and it would appear that the large molecule or colloidal particle acts merely as a carrier of the haptene, anchoring it, as it were, in the tissues of the animal, and thus creating sufficient opportunity for antibody to be developed. It seemed possible that the flocculable antigen present in the Seitz filtrates of vaccinia suspensions might be turned into a complete antigen by adsorbing it on inert colloidal particles like collodion, and in order to see whether this were so or not the following experiments have been made.

DESCRIPTION OF TECHNIQUE.

Virus.

The strain of vaccinia used was derived from calf lymph (Lister Inst. No. 1715) and passed on the shaved skin of rabbits. Crusts were ground, suspended 1 : 10 by moist weight in 50 p.c. glycerin-saline containing brilliant green 1 : 10,000, and stored overnight in the ice-chest. Next day the back and flanks of a white-skinned rabbit were shaved with the minimum of trauma, and the inoculum thoroughly rubbed in with the rubber-gloved hand. Vesicles began to appear on the 2nd to 3rd day. On the 5th to 7th day, when the lesions were well crusted, the rabbit was killed, the inoculated skin was removed, pinned out on a board and scraped with a sharp Volkmann's spoon. The material obtained (3 to 6 g.) was frozen and stored till required.

Preparation and Filtration of Vaccinial Suspensions.

A suitable quantity of crusts was ground and suspended in ten times its weight of saline. After sedimenting overnight in the refrigerator the suspension was further clarified by centrifugation at a moderate speed for one hour, and the clarified supernatant filtered through a Seitz E.K. filter at a negative pressure of 300 mg. Hg. The filter was treated beforehand by filtering 10 c.c. of 1 in 10 rabbit serum in saline, followed by 10 c.c. saline. The filtrate was tested for bacterial sterility by sowing in broth and on agar, for virulence by inoculation on the scarified skin of a guinea-pig's flank, and for flocculating

power against an anti-vaccinial serum of known titre. Only samples of filtrate which were bacteria-free, non-virulent, and showed a satisfactory flocculating titre were used in the following experiments.

Preparation of Collodion Particles.

The method used was based on that described by Zozaya (1932). 10 g. of pure dry collodion (pyroxylin B.D.H.) was added to a litre of pure acetone in a well-stoppered bottle, and allowed to stand, with occasional shaking, till dissolved (several days). 100 c.c. of this stock solution was put into a litre flask, and diluted five times with acetone. A jet of distilled water was directed into the flask, which was shaken vigorously, and continued until the whole became milky. Further distilled water was added, up to a total volume of about 800 c.c. The mixture was poured into a 2-litre distilling flask, and distilled under reduced pressure at 60° C. till bubbling became intermittent. The residue was poured into an evaporating basin, and cautiously boiled over a small flame till there was no more smell of acetone. The residue in the basin was poured into a tall glass cylinder and allowed to stand for 5 minutes, then cautiously poured into another cylinder, so that any large lumps were left behind. The process was repeated with successive lots of stock collodion solution, till about a litre of watery suspension had been collected. To this 0·9 p.c. NaCl was added. The collodion particles settled out overnight and the supernatant fluid was siphoned off, leaving a thick creamy deposit, which was re-suspended in 0·9 p.c. saline. This was repeated till all trace of smell and taste of acetone was gone. The final gravity-deposit was transferred to a flask, and steamed for 20 minutes on three successive days.

The amount of collodion present in unit volume of this suspension was measured by centrifuging a known volume and weighing the dried deposit. When examined under the microscope the suspension was seen to consist of highly refractile particles, which appeared of remarkably regular spherical shape and uniform size, and approximately 0·5μ in diameter. In saline these were clumped together in large irregular groups, but in distilled water they were discrete.

Method of Adsorption.

Sufficient of this stock of collodion particles to make a 4 p.c. suspension in the volume of Seitz filtrate to be adsorbed was centrifuged and the supernatant saline decanted. The filtrate was poured on to the deposited collodion, shaken till the particles were dispersed and stored overnight in the ice-chest. Next day the particles were centrifuged down, and the supernatant fluid decanted and kept for a flocculation test. The particles were resuspended in saline, well shaken and centrifuged again. The supernatant saline was decanted, and also kept for a flocculation test. The particles were resuspended in a volume of saline equal to that of the filtrate adsorbed, and this suspension formed the inoculum. The filtrate, after adsorption, was never found to retain more than 10 p.c. of its original flocculating power. The saline washings were generally devoid of flocculating power.

The Flocculation Reaction.

The method used was the same as that described by Craigie (1932*a*), with certain modifications. When the antigenic content of a filtrate was to be tested, an equal volume (0·2 c.c.) of a constant dilution of anti-vaccinia rabbit serum was added to each of a series of three-fold dilutions of the antigen, a control series being put up with normal rabbit serum. When sera were to be tested for antibody, an equal volume (0·2 c.c.) of a constant dilution of a good flocculating Seitz filtrate was added to each of a series of two-fold dilutions of each serum, of normal rabbit serum, and of an antivaccinial serum of known titre. The constant dilutions of anti-vaccinia serum and of Seitz filtrate chosen for use in these two types of test respectively were the highest dilutions which gave maximum flocculation. For anti-vaccinia serum this was 1 : 10 or 1 : 20, and for the filtrate 1 : 60. Controls of antigen + saline and serum + saline were always included. From time to time control antigens prepared from a rabbit's skin strain of herpes febrilis were also included.

The test was kept at 4° C. in the ice-chest for 40 hours, allowed to warm up to room temperature, and read with the help of indirect light and a hand lens. The advantages of performing the test at 4° C. instead of 50–55° C. are that anomalous flocculation does not occur, and also that the inhibition of flocculation by fresh serum described by Craigie (1932*b*) is not met with. Parallel tests made by the two methods gave similar readings.

Neutralizing Power of Sera.

Equal volumes of each serum to be tested and of normal rabbit serum were added to identical series of ten-fold dilutions of a virus suspension. After standing for 2½ hours at room temperature, the mixtures were inoculated by scarification on the shaved flanks of guinea-pigs. Results were read on the 3rd, 4th, 5th and 6th days. The virus-neutralizing power of a serum was measured by the reduction in the titre of the virus, expressed in minimal infective doses, which it effected under these conditions.

Tests of Immunity.

Identical series of ten-fold dilutions of a virus suspension were made and inoculated by scarification on the flanks of each of the rabbits to be tested, and of a normal rabbit. Immunity was measured by the difference in the titre of the virus in the rabbit being tested and on the normal rabbit, expressed in minimal infective doses.

Production of Anti-vaccinia Serum.

The method recommended by Burgess, Craigie and Tulloch (1929) was at first adopted, *i. e.* ten or more weekly injections of living virus, the first four or so intravenous, the later ones intraperitoneal. It soon became evident that flocculation was at its optimum after the second or third intravenous injections and, indeed, sometimes declined after further injections. A short course was therefore used, often consisting of a skin inoculation followed by two weekly intravenous injections. Samples of serum were chosen, for use as controls in the following experiments, which gave perceptible flocculation at a dilution of 1 : 32 in the presence of a 1 : 60 Seitz filtrate.

EXPERIMENTAL RESULTS.

Experiment 1.

This preliminary experiment was planned as a qualitative test of the antigenic power of vaccinial Seitz filtrates, and of the effect on this power of adsorption on collodion particles. Four rabbits were inoculated intravenously according to the plan followed by Zozaya (1932). Two of them, F27 and F28, received Seitz filtrate ; the other 2, C29 and C30, received a suspension of collodion particles which had been treated with the same amounts of the same Seitz filtrate. On 3 successive days 0·5 c.c., 1 c.c. and 1 c.c. of Seitz filtrate were injected into F27 and F28 and particles into C29 and C30. After 7 days' interval 1 c.c., 2 c.c. and 2 c.c. were injected on 3 successive days, and after a further 14 days' interval 2 c.c., 2 c.c and 3 c.c. on 3 successive days. The rabbits were bled 7 days after each group of 3 injections and at intervals afterwards, and the sera were tested for antibody content by the flocculation reaction.

C29 and C30 were the first to develop flocculating antibody. It appeared in sera collected after the second group of 3 injections of treated collodion particles, and rose to its height after the third group of injections, when its titre was equal to that of a good anti-vaccinia serum.

F27 and F28 developed no flocculating antibody till after the third group of 3 injections of Seitz filtrate, and in later samples of serum its titre did not rise higher than half that of a good anti-vaccinia serum.

F28 and C30 were tested for immunity, by titration of a vaccinial suspension on the shaved scarified skin of the flank, 2 months after the last injections. C30 showed an ungraded pink swelling of all the test areas, which appeared before 24 hours and lasted about 48 hours—clearly an allergic reaction. F28 showed no trace of this. Both rabbits proved immune to approximately 100 M.I.D.—an immunity which, though not quite complete, was not surpassed by the rabbit immunized with living virus used as a control. Sixteen days later F28 and C30 were bled out under ether. In the serum of F28 flocculating antibody had fallen almost to *nil*, while in that of C30 it was still at full titre.

F27 and C29 were kept for a longer period before being tested for immunity. Tested as above 4 months after the last injections, they both showed a doubtful immunity to only 10 M.I.D. with modified lesions, consisting of a small vesicle or group of vesicles surmounting a wide, raised, sometimes dusky-red areola, and no allergic reaction.

Experiment 2.

In order to bring out more clearly the difference in antigenic power between Seitz filtrates alone and when adsorbed on collodion particles, a more gradual immunization was carried out.

Two rabbits were given 4 fortnightly intravenous injections. F51 received 2 c.c. of Seitz filtrate ; C53 received collodion particles treated with the same amount of Seitz filtrate. They were bled 9 to 12 days after each injection ; the sera were tested for flocculating power as before, and also for virus-neutralizing power. After 2 injections had been given, C53 showed marked antibody

response, but F51 still showed none ; while after the fourth injection C53 still maintained its lead, though F51 had begun to respond. Immunization was stopped at this point, in the hope that the two rabbits would show as marked a difference in immunity as they had done in antibody production. However, when they were tested for immunity a fortnight after the last injections, their behaviour was very similar. Both gave an allergic reaction, more marked in C53 than F51, and a doubtful immunity to only 10 M.I.D.

These results are given in Table I.

TABLE I.

Date.	Seitz filtrates. Intravenous injections.	Rabbit F51. F.	N.	I.	Date.	Seitz filtrates adsorbed on collodion particles. Intravenous injections.	Rabbit C53. F.	N.	I.
1.v.34	..	0	..	..	12.iv.34	..	0	..	..
18.v.34	2 c.c.	..	..	..	4.v.34	2 c.c.	..	..	..
31.v.34	..	0	0	..	14.v.34	..	4	10	..
4.vi.34	2 c.c.	..	..	..	18.v.34	2 c.c.	..	..	..
13.vi.34	..	0	0	..	31.v.34	..	8	100	..
18.vi.34	2 c.c.	..	..	..	4.vi.34	2 c.c.	..	..	..
28.vi.34	..	4	10	..	13.vi.34	..	16	100	..
3.vii.34	2 c.c.	..	..	..	18.vi.34	2 c.c.	..	..	..
13.vii.34	..	4	10	..	28.vi.34	..	32	100	..
17.vii.34	..	..	..	10	3.vii.34	..	..	..	10A

F. = Flocculation reaction. The number is the reciprocal of the highest dilution of serum showing flocculation. N. = Neutralization test. Expressed in number of M.I.D. of virus neutralized. I. = Immunity test. Expressed in number of M.I.D. to which the animal was immune. A. = Allergic reaction. M. = Modified lesion. The same notation is used in Table II.

Experiment 3.

A further experiment on similar lines was undertaken, in which 6 rabbits were given weekly intravenous injections. Rabbits F135, F136 and F137 each received 2 c.c. of Seitz filtrate, and C132, C133 and C134 received the same filtrates adsorbed on collodion particles. Each rabbit was bled a week after each injection (*i. e.* immediately before receiving the next injection), and the sera were tested for flocculating and for virus neutralizing power. A week after the third injection F137 and C134 were tested for immunity. The remaining rabbits received one more injection each, and a week later they, too, were tested for immunity.

The results, which are given in Table II, confirmed and amplified those of Experiments 1 and 2. Again, it was after the second injections that the contrast between the two groups of rabbits was most plainly shown, antibody production being abundant in one, negligible in the other. Later this contrast was to some extent obscured by individual differences and by apparent anomalies in the results of the immunity tests. Tested after 3 injections, G34 was strongly immune, F137 only very slightly so, agreeing well with their antibody production. But after 4 injections, C132 was only moderately and C133 very slightly immune (both showing modified lesions), while F135 was strongly immune and F136 slightly so—results which show little correspondence with antibody production.

TABLE II.

Date.	Injections.	Seitz filtrates.									Seitz filtrates adsorbed on collodion particles.								
		Rabbit F135.			Rabbit F136.			Rabbit F137.			Rabbit C132.			Rabbit C133.			Rabbit C134.		
		F.	N.	I.	F.	N.	I.	F.	N.	I.	F.	N.	I.	F.	N.	I.	F.	N.	I.
13.viii.34	..	0	..	..	0	..	..	0	..	..	0	..	..	0	..	..	0	..	..
	2 c.c.	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
21.viii.34	..	0	10	..	2	0	..	0	10	..	0	0	..	0	10	..	4	10	..
	2 c.c.	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
28.viii.34	..	2	0	..	0	0	..	0	10	..	32	10	..	8	100	..	8	10	..
	2 c.c.	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
4.ix.34	..	16	10	..	4	10	..	4	10	10	32	..	..	32	..	..	32	100	1000
	2 c.c.	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
10.ix.34	..	32	1000	..	8	100	..	..	..	..	32	1000	..	32	1000	..	..	..	..
11.ix.34	..	..	..	1000	..	..	10	..	..	..	..	..	100 M.	..	..	10 M.	..	..	..

THE HEAT-STABILITY OF THE FLOCCULABLE SUBSTANCE.

As was mentioned in the first section, the flocculable extract prepared by Wilson Smith (1932) from autolysed vaccinial rabbit testicle withstood repeated boiling. Craigie, however, found that his Seitz filtrates of vaccinial rabbit-skin crusts were labile at 55° C.

In the course of the present work many samples of Seitz filtrate of vaccinial rabbit-skin crusts were tested for heat stability. They were first acidified to pH 5·4 with acetic acid and then boiled for 3 minutes. The heavy precipitate which formed was centrifuged down, and the supernatant neutralized with NaOH, and tested for flocculating power. As a rule all trace of flocculating power was destroyed by this procedure, but in an occasional sample it survived, sometimes almost undiminished. For a long time the cause of these discrepancies remained obscure, though every likely factor was investigated in turn. Eventually it was found that when a fresh suspension (not more than 24 hours in the ice-chest) of vaccinial crusts was filtered, the flocculating power of the filtrate was completely destroyed by boiling for 3 minutes. But when the suspension was allowed to stand for a week or more in the ice-chest or, better still, for 3 to 5 days in the incubator at 37° C. before filtration, the flocculating power of the filtrate was little, if at all, reduced by boiling for 3 minutes.

Consideration of these facts suggested the possibility of preparing a protein-free flocculable extract, and of using it in inoculation experiments analogous to the foregoing. It was thought that such an extract was likely to be devoid of antigenic power in the strict sense, and thus might provide a means by which the effect of adsorption upon a colloidal carrier could be demonstrated even more convincingly.

It has so far proved impossible, however, to remove all protein by any simple process. The boiled filtrates always contained traces of protein. They were unsatisfactory also in that their content of flocculable substance was variable, and they sometimes developed a non-specific opacity in contact with serum which obscured the flocculation reaction.

Protein precipitants such as sulphosalicylic acid or trichloracetic acid removed or destroyed the flocculable substance.

DISCUSSION.

In order to estimate correctly the relative significance of these results, certain points must be borne in mind.

The flocculation reaction is found in practice to be reliable and accurate. Titrations of two-fold dilutions of a serum can be carried out repeatedly with a very fair degree of correspondence. But the estimation of virus-neutralizing power, depending as it does on a biological test, is far less accurate. Tenfold dilutions of virus are the closest that can usefully be employed, and even then random variations occur. Similar inaccuracies affect the immunity test, and here natural variations in susceptibility cause added uncertainty. Moreover, in experiments of this kind only one immunity test can be made on each animal, and it is often impossible to tell whether immunity is on the up or down grade at the time of the test.

The significance of allergic reactions and of " modified lesions " is not very clear. Most probably they occur at stages of partial immunity. They are not shown by the fully immune animal.

It is evident that reliance must be placed chiefly on the results of the flocculation reaction ; those of virus neutralization and immunity tests should be regarded as auxiliary only.

The results of Experiments 1, 2 and 3, taken together, leave little doubt that a Seitz filtrate of vaccinial rabbit-skin crusts is a weak antigen, and that a suspension of collodion particles which has been in contact with such a filtrate is more strongly antigenic (*i. e.* produces an equal effect with fewer injections) than the filtrate itself.

The flocculation reaction supports these conclusions consistently in the three experiments. Virus neutralization runs approximately parallel with flocculation. When the immunity tests are considered it is seen that they show divergencies, already referred to, which their inherent inaccuracy does not altogether account for. In this connection it is of interest to note that during a course of injections of living virus the immunity of a rabbit may at first rise to a high value and then fall off again. It appears likely that something of the kind has happened in these experiments. For instance, in Experiment 3 it is possible that if all the rabbits had been tested for immunity at the same time as C134 and F137, all the " C's " would have shown strong and all the " F's " weak immunity.

It may be conjectured that the extra injection had the effect of lowering the immunity of the already highly immunized C132 and C133, while it raised that of F135 to its maximum, and slightly raised that of F136, which had not nearly reached its maximum. In any case, the number of rabbits tested was too small for a definite decision to be reached on this question.

The full significance of the observed enhancement of antigenic power brought about by adsorption on collodion particles becomes clear when the experimental methods are recalled. The particles, after being in contact with the filtrate, were thoroughly washed and resuspended. If their subsequent antigenic power had merely been that of the filtrate which they retained, it could not have been greater and would probably have been less than that of the filtrate alone. The superiority which they, in fact, showed must be regarded as significant.

The antigenicity of Seitz filtrate does not, of course, prove the antigenicity of the flocculable substance itself. To prove this it would be necessary to obtain the flocculable substance free of extraneous protein.

The chemical nature of the flocculable substance remains undecided. In the state of impurity in which it is at present available its properties depend on the methods by which it has been prepared.

CONCLUSIONS.

1. Seitz filtrates of suspensions of vaccinial rabbit-skin crusts possess some antigenic power.

2. This power can be definitely enhanced by adsorption on collodion particles.

3. A heat-stable flocculating substance may be prepared from vaccinial rabbit's-skin crusts similar in properties to that obtained by Wilson Smith from vaccinial rabbit's testicle.

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THE AGGLUTINOGENS OF A STRAIN OF VACCINIA ELEMENTARY BODIES.

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OBSERVATIONS on the agglutination of the elementary bodies of vaccinia have been reported by Ledingham (1931, 1932, 1933), Craigie (1932) and by Amies (1932). In a brief communication (Craigie, 1934) it was reported that killed* vaccinia elementary bodies stimulated the production of agglutinins in rabbits isolated from accidental infection with living virus. When the killed elementary bodies were injected intravenously the agglutinin response to one injection was marked and prompt, but further intravenous injections were required to produce complement-fixing antibodies and precipitins in demonstrable amounts. On the other hand, after inoculation with infective

* The term "killed" as used here signifies that after the elementary body suspension had been subjected to treatment designed to kill the virus, no vaccine virus could be recovered from the suspension by combined serial testicular and intradermal transfers.

elementary body suspensions, complement-fixing antibodies appear simultaneously with, or 24 hours later than, the agglutinins, and subsequently there is a fairly constant degree of parallelism between the agglutinin and complement-fixing titres.

This difference in the antibody response of rabbits to infective and to killed elementary body suspensions and the difficulty of immunizing with dead vaccinia virus* redirected attention to the possibility that the elementary bodies of vaccinia might present an antigenic complexity. An attempt had previously been made to determine whether the elementary bodies of vaccinia contained more than one agglutinogen, but the results obtained were not encouraging. Reviewing these unsuccessful experiments in the light of present knowledge, it appears that unsuitable sera and methods of preserving and treating the elementary bodies were responsible for the negative results obtained. In view of this, a detailed description of the materials and methods used is presented.

The purpose of this communication is to present evidence that a strain of elementary bodies (C.L.) contains two agglutinogens, one of which is relatively heat-stable. In doing so no attempt will be made to indicate the progress of the investigation, but, after presentation of the evidence, reference will be made to certain difficulties which were encountered during its course.

METHODS AND TECHNIQUE.

Strain of vaccinia elementary bodies.—This strain, designated C.L., was derived from a calf strain (originally New York Dept. of Health) employed for the production of vaccine lymph. Since transfer to the rabbit it has been propagated on the skin solely by scarification, and for the past 2½ years washed elementary body suspensions have been employed in transferring the virus from rabbit to rabbit.

Production of elementary body suspensions.—Bronze wire gauze (100-mesh) is employed for inoculation of the closely-clipped skin of the rabbit. A strip of size 0·7 × 10 cm. is torn off, folded lengthwise in four and grasped in Kocher forceps. The rough edges of the gauze are used to lightly scarify the inoculum into the skin. The elementary bodies are harvested three days after inoculation. After the rabbit has been exsanguinated, the inoculated skin is removed, pinned out on a level board and cleansed with ether. The pulp is scraped off into two successive 10 c.c. volumes of chilled dilute buffer† placed on the skin, a blunt scalpel being used to remove the pulp. After transfer of the pulp suspension to a centrifuge tube, all subsequent centrifugalization is carried out in the cold room. The pulp suspension is not ground up, since this is unnecessary, but after thorough shaking is centrifuged immediately at 3500 r.p.m., horizontally, for 10 minutes. The supernatant is set aside, and the pulp again shaken up in dilute buffer and centrifuged. The two supernatants are pooled and again centrifuged. The supernatant containing the elementary bodies is transferred to flat angle centrifuge tubes (4 mm. internal diameter), and centrifuged for one hour (3500 r.p.m.) at an angle of 28° from the vertical.

* *Vide* Bland (1932).

† McIlvaine's citric acid-phosphate buffer pH 7·0 diluted 1 in 50 in distilled water.

The deposited elementary bodies are resuspended and subjected to two further washings in dilute buffer in the angle centrifuge. The final suspension is dispersed by thorough shaking, and given a final centrifugalization in the centrifuge used for the preliminary removal of tissue and cells. The supernatant containing the washed elementary bodies is placed in glass-stoppered bottles, an excess of anæsthetic ether is added and the mixture thoroughly shaken. When required for use a sufficient portion of suspension is pipetted out from under the ether, and the ether dissolved in the suspension is removed in a partial vacuum. The yield of elementary bodies from a rabbit is taken up in 15–20 c.c. of dilute buffer. This concentration, when fresh, usually has an intradermal titre of approximately 1 in 10^8, and is 20 to 40 times the standard density referred to below.

Antivaccinia serum.—The sera employed were obtained from rabbits (*a*) 10 to 14 days after cutaneous vaccination and (*b*) 4 to 6 days after the intravenous injection of infective elementary body suspension into immune rabbits. After separation of the cells the sera were heated at 56° C. for 45 minutes and stored without preservative.

Heat treatment of elementary body suspensions.—The conditions under which the suspension is heated are important, since instability is readily produced by heat. If the elementary body suspension is heated undiluted in dilute buffer 0·0036 M, pH 7·0 to 7·2, instability rarely results.

Absorption of agglutinins.—The estimated minimal complete absorbing dose of elementary body suspensions (in dilute buffer) is first added to the required volume of serum. To this mixture the requisite amounts of distilled water and 8·5 p.c. NaCl solution is added to dilute the serum to four times the strongest concentration required in the subsequent agglutination test and to bring the concentration of NaCl to 0·85 p.c. After the mixtures have stood at room temperature for one hour they are incubated for one hour at 37° or 50° C. and then centrifuged. Unless an excess of elementary bodies has been added, the angle centrifuge is not required to clear the supernatant. Absorption occurs rapidly, and more prolonged contact of the serum with the elementary bodies offers no advantages. The approximate minimal absorbing dose may be calculated from the density of the elementary body suspension and the agglutinin titres of the serum, which have been ascertained with suspensions of standard density. The following formula is employed: Volume of suspension required $= \frac{Vy}{2xD}$, where V = volume of serum, y = titre of serum, x = dilution of serum required, and D = concentration of the elementary body suspension expressed as a multiple of the standard density. If there is doubt concerning the adequacy of the absorbing dose, less distilled water and saline than ultimately required are added to the mixture of serum and suspension and it is observed during the first hour. Should the dosage of elementary bodies be insufficient, agglutination is rapid and the supernatant becomes clear; in this event a further amount of suspension is added.

Agglutination test.—The series of serum dilutions (0·25 c.c.) is made in 0·85 p.c. NaCl containing 1 : 50 citric acid, phosphate buffer pH 7·0. The elementary bodies are diluted to twice the standard density in distilled water containing 1 : 50 of this buffer, and 0·25 c.c. added to each serum dilution.

In diluting the suspension to the required density a kaolin standard is employed. This standard was prepared from a suspension of kaolin from which the larger particles had been allowed to settle out for several days, and was chosen since its appearance in varying proportions of transmitted and reflected light closely matches that of an elementary body suspension. The kaolin standard, twice the standard density, matches tube No. 2 of the McFarland standard when they are compared against a dark background and illuminated from behind at an angle of 45°. The agglutination tests are incubated overnight (16 hours) in a water-bath of 50° C.

EXPERIMENTAL.

On inquiring into the effect of heat on the elementary bodies of vaccinia, it was found that stable heated suspensions of elementary bodies retained their agglutinability, although, with most sera, they indicated a slightly lower agglutinin titre. When heated suspensions were employed to absorb such sera results similar to those shown in Table I were obtained, the heated suspension absorbing agglutinins for itself without exerting any comparable effect on the agglutinins for unheated suspension.

TABLE I.—*Absorption of Serum No.* 216 *with Unheated and Heated Elementary Bodies.*

Treatment of serum.	E.B. suspension.	Dilutions of serum. 1/10	1/20	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.
Not absorbed	N	+++	+++	+++	+++	+++	++	+	−
	H	+++	+++	++	++	+	−	−	−
Absorbed with N	N	+	−	−	−	−	−	−	−
	H	−	−	−	−	−	−	−	−
Absorbed with H	N	+++	+++	+++	+++	++	+	tr.	−
	H	−	−	−	−	−	−	−	−

N = Unheated elementary body suspension No. 279.
H = E.B. suspension No. 279 heated at 90° C. for 1 hour.

Further investigation showed that a temperature of 90° C. was greatly in excess of that required to inactivate the less stable component as regards absorption and agglutination. In a large number of experiments a temperature of 56° C. for 45 minutes has been sufficient. Table II is representative of the results obtained in absorption tests with elementary bodies heated to temperatures from 56° to 95° C., and which failed to show any agglutinative or absorptive differences between suspensions heated at temperatures within this range.

Results similar to those indicated in Tables I and II have been consistently obtained with all elementary body suspensions and sera examined since the technique described above was adopted. It is clear that rabbit antivaccinia serum, obtained 10 to 14 days after vaccination or subsequent to a further course of inoculation with living virus, contains two agglutinins. The corresponding agglutinogens differ markedly in their stability to heat under the conditions defined above. One of these is inactivated, as far as agglutinability and capacity to absorb is concerned, by exposure for 45 minutes to temperatures

over 56° C. We propose to designate this the " L agglutinogen ". The other resists temperatures up to 95° C. and will be referred to as the " S agglutinogen ".

TABLE II.—*Absorption of Serum No. 231 with Elementary Body Suspension No. 279 Heated at Different Temperatures.*

Treatment of serum.	E.B. suspension.	Dilutions of serum. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.
Not absorbed	N	+++	+++	+++	+++	++	+	−
	H_1	+++	+++	+++	+++	+	tr.	−
	H_2	+++	+++	+++	+++	+	tr.	−
	H_3	+++	+++	+++	+++	+	tr.	−
Absorbed with N	N	tr.	−	−	−	−	−	−
	H_1	tr.	−	−	−	−	−	−
	H_2	tr.	−	−	−	−	−	−
	H_3	tr.	−	−	−	−	−	−
Absorbed with H_1	N	+++	+++	+++	+++	++	+	−
	H_1	tr.	−	−	−	−	−	−
	H_2	tr.	−	−	−	−	−	−
	H_3	tr.	−	−	−	−	−	−
Absorbed with H_2	N	+++	+++	+++	+++	++	+	−
	H_1	tr.	−	−	−	−	−	−
	H_2	tr.	−	−	−	−	−	−
	H_3	tr.	−	−	−	−	−	−
Absorbed with H_3	N	++	+++	+++	+++	++	+	−
	H_1	tr.	−	−	−	−	−	−
	H_2	tr.	−	−	−	−	−	−
	H_3	tr.	−	−	−	−	−	−

N = Unheated E.B. snspension.
H_1 = E.B. suspension heated at 56° C. for 45 minutes.
H_2 = E.B. suspension heated at 70° C. for 45 minutes.
H_3 = E.B. suspension heated at 90° C. for 45 minutes.

The minimum temperature of inactivation of the L agglutinogen (in 45 minutes) may vary slightly in different suspensions, or even on repetition with the same suspension. It lies between 52° and 56° C. The almost complete inactivation of this agglutinogen at 52° C. is shown in Table III.

TABLE III.—*Impairment of L Agglutination of Elementary Body Suspension No. 64 Heated below 56°.*

Temperature at which E.B. suspension was heated for 1 hour.	Serum No. 245 after absorption with heated elementary bodies. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.
56° C.	−	−	−	−	−	−	−	−
54° C.	(tr.)	?	−	−	−	−	−	−
52° C.	tr.	(tr.)	(tr.)	tr. ?	−	−	−	−
Not heated	+++	+++	+++	+++	++	+	tr.	−

On the other hand, a sample of suspension showing incomplete inactivation at 56° C. has been encountered several times, but this result was not obtained on heating further samples of the same suspensions on subsequent occasions. Inactivation may occur at temperatures below 50° C. provided the period of

TABLE IV.—*Influence of Formalin on the Agglutinability of Elementary Bodies.*

Elementary body suspension.	Serum No. 165. Not absorbed. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	Absorbed with heated E.B. suspension. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.
No. 206. Unheated	+++	+++	+++	++	+	−	+++	+++	++	+	−	−
No. 206, Heated	+++	+++	+++	++	+	−	−	−	−	−	−	−
No. 77, Formalinized	+++	+++	+++	++	+	−	−	−	−	−	−	−
No. 101, ,,	+++	+++	+++	++	+	−	+	+	tr.	−	−	−
No. 99, ,,	+++	+++	+++	++	+	−	+	+	tr.	−	−	−

No. 206 = Heated at 70° C. for 30 minutes.
No. 77 = Stored with 1 p.c. formalin pH 7·0.
Nos. 101 and 99 = Stored with 0·25 p.c. formalin pH 7·0.

TABLE V.—*Influence of Methylene-Blue and Eosin Y on the Agglutinability of Elementary Body Suspension No. 235.*

E.B. suspension.		Serum No. 166. Not absorbed. 1/20.	1/40.	1/80.	1/160.	1/320.	Absorbed with heated E.B. suspension. 1/20.	1/40.	1/80.	1/160.	1/320.
Control	L	+++	+++	++	+	−	+++	++	+	tr.	−
	D	+++	+++	++	+	−	+++	++	+	tr.	−
With methylene-blue 1 in 10^4	L	+++	++	+	tr.	−	tr.	−	−	−	−
	D	+++	+++	++	+	−	+++	++	+	tr.	−
With eosin Y 1 in 10^5	L	+	tr.	−	−	−	−	−	−	−	−
	D	+++	+++	++	+	−	+++	++	+	tr.	−

L = Suspension exposed to diffused daylight for 5 hours.
D = Suspension kept in dark for 5 hours.

exposure is prolonged. Even at 37° C. for 24 hours some impairment of the agglutinability of the suspension in a pure L serum occurs. Exposure of a suspension under ether for 10 days at 37° C. resulted in complete loss of agglutinability in a pure L serum, but suspensions stored under ether at + 4° C. for periods up to a year have shown no obvious loss of L agglutinogen and retain a high degree of infectivity. The inactivation of the L agglutinogen at 56° C. has been observed to occur as readily under completely anaërobic conditions as in open tubes.

The S agglutinogen is manifestly stable at temperatures up to 95° C. At 100° C. some impairment of its agglutinability may result, but its absorptive capacity does not appear to be affected.

Agents other than heat have been observed to exert a selective destructive action on the L agglutinogen. In the presence of formalin partial or complete inactivation of the agglutinability of this component may occur (Table IV), but this effect, when partial, may be complicated by the tendency of formalin to render the suspension hyperagglutinable or actually unstable. The suspensions referred to in Table IV did not show this hyperagglutinability.

Methylene-blue, in the presence of light, selectively inactivates the L agglutinogen. It is of interest that eosin Y, which in the presence of light not only kills vaccine virus, but inactivates the flocculable substance of vaccinia (Craigie and Tulloch, 1931), has an effect on the S as well as the L agglutinogen (Table V).

Although the L agglutinogen is labile at 56° C., we failed to obtain any evidence that L agglutination is impaired at this temperature. Duplicate tests in which L agglutination at 50° and 56° C. were compared showed identical results. Other tests carried out at various temperatures showed that for both L and S agglutination a temperature as high as 50° C. was required for complete agglutination. It would appear that the union of L agglutinin with the corresponding agglutinogen is sufficiently rapid to obviate false results, even if the tests are placed in the 56° C. water-bath immediately after mixing.

A brief indication of the factors which appear to have contributed to the failures of our earlier attempt to obtain evidence of a labile antigen in the elementary bodies of vaccinia may be of some value. Formalin had been chosen as a preservative for elementary body suspensions destined for agglutination tests since it seemed to make agglutination actually more clear-cut. Suspensions preserved with formalin frequently gave complete agglutination in serum dilutions in which they agglutinated incompletely when formalin was omitted, and also showed a sharper end-point and a rather higher titre for the serum. Now, as has been shown above, formalin actually damages the L agglutinogen, but, on the other hand, it tends to increase instability of the suspension. This instability may not be sufficient to cause any precipitation of the elementary bodies in the control tube, yet it would seem to render the suspension unstable in dilutions of serum which would not otherwise cause agglutination. Formalin-treated suspensions yielding an exaggerated agglutinin titre for a serum have been observed shortly afterwards to become unstable in 0·85 p.c. NaCl. Partial destruction of L agglutinogen by formalin would seem to be more or less compensated, under certain conditions, by increased

sensitivity to L agglutinins. This view is supported by the observation that many multiples of the minimal absorbing dose given by the formula $\frac{\text{“}Vy\text{”}}{2xD}$ are required for absorption of a serum when a formalin-treated suspension is used.

It has been found that addition of sodium chloride to elementary body suspensions is to be avoided until the absorption or agglutination test is set up, and in the agglutination test its concentration should be reduced to 0·425 p.c. or less. The presence of sodium chloride during heating of the suspension often promotes instability. Brief exposure of elementary bodies to strong NaCl solutions renders them permanently unstable. No method of restabilizing an unstable suspension has been found.

Another important factor in the earlier failure to demonstrate the L agglutination was undoubtedly the types of serum employed. Most of those available at the time were produced by the inoculation of vaccinated rabbits with formalin-treated elementary bodies or Seitz filtrates containing the specific precipitable substance of vaccinia.

DISCUSSION.

The marked lability of the L agglutinogen of the C.L. strain of vaccinia elementary bodies is of interest in view of experience in the past of the feeble or non-existent immunizing value of killed vaccine virus. We have experienced considerable difficulty in rendering elementary body suspensions non-infective, without either manifestly destroying the agglutinability of the L agglutinogen or rendering the suspension too unstable for agglutination tests. It should not, however, be assumed that this labile agglutinogen is a factor in immunity to vaccinia virus, and that its lability provides an explanation of the immunizing deficiencies of killed vaccine virus. With equal justification it might be suggested that vaccine virus contains another labile antigen, the antigenic integrity of which is bound up with the life of the virus. It is, however, premature to pursue this question further, or to consider the view that immunity to vaccine virus is entirely dependent on the persistence of the virus in the animal body. Reference is required, however, to the Vi antigen of *B. typhosus* recently reported on by Felix and Pitt (1934). The Vi agglutinogen described by these authors is probably distinct from the rather more stable Vi antigen responsible for the production of immunity in mice to a virulent strain of *B. typhosus*, and resembles the L agglutinogen of the elementary bodies of vaccinia in its lability to treatment which is lethal for the organism. The resemblance between these two agglutinogens may extend no farther than this, but even so, it is an important point, and it is not improbable that antigens of this exceedingly labile type await recognition in other organisms.

CONCLUSIONS.

1. Agglutinating sera for the elementary bodies of vaccinia, obtained after the vaccination of rabbits, with or without further inoculation of elementary bodies of the C.L. strain of vaccine virus, contain two agglutinins.

2. The two corresponding agglutinogens differ in their relative stability to heat and other agents.

3. The more labile L agglutinogen has its agglutinability and ability to absorb agglutinin impaired or destroyed by exposure to a temperature as low as 56° C.

4. The S agglutinogen is stable at temperatures up to 95° C.

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THE CORTICAL LIPOID OF THE SUPRARENAL.

WE have received the following correction from Dr. Raymond Whitehead with a request for its publication:

"Dr. Ruth Deanesly points out that my reference on p. 284 of the current volume of the *British Journal of Experimental Pathology* to the observations on cortical lipoid contained in her paper in the *American Journal of Anatomy* (1931, vol. xlvii, p. 475) is inaccurate in that it implies that all the mice were unilaterally suprarenalectomized. Actually only 24 of the total 104 mice were so treated. Dr. Deanesly's paper contains many valuable observations concerning the histological reaction of the suprarenal as a whole, upon which no criticism was intended. I can only express my sincere regret that my published statement fails to make this clear."

PAUL FILDES,
General Editor.

INDEX TO AUTHORS.

ALPORT, A. C., the treatment of septicæmia in rabbits with lymph-gland fixation abscesses, 175

AMIES, C. R., the attempted cultivation of vaccinia virus in conjunction with non-pathogenic micro-organisms, 185

——— the elementary bodies of zoster and their serological relationship to those of varicella, 314

——— the influence of temperature on the survival of pure suspensions of the elementary bodies of vaccinia, 180

ASCHNER, M., see *Kligler, I. J.*

BEDSON, S. P., and BLAND, J. O. W., the developmental forms of the psittacosis virus, 248

BELL, G. H., and MORRIS, S., the oxytocic properties of the blood and its relation to milk fever in cows, 143

BERENBLUM, I., and KENDAL, L. P., the production of tumours in the fowl with a colloidal solution of 1 : 2 : 5 : 6-dibenxanthracene, 366

BEST, C. H., see *MacLean, D. L.*

BHATNAGAR, S. S., see *Felix, A.*

BLAND, J. O. W., see *Bedson, S. P.*

BORTHWICK, GRISEL R., some factors predisposing to infection by *Vibrion septique* from the alimentary tract, 153

BROWN, H. C., see *Findlay, G. M.*

BURNET, F. M., antigenic differences between related bacterial strains: a criticism of the mosaic hypothesis, 354

——— the propagation of the virus of infectious laryngo-tracheitis on the chorio-allantoic membrane of the developing egg, 52

——— and FERRY, J. D., the differentiation of the viruses of fowl plague and Newcastle disease: experiments using the technique of chorio-allantoic membrane inoculation of the developing egg, 56

BUTTLE, G. A. H., attempts to locate the site of antibody production, 64

CAMPBELL, J. A., cancer of skin and increase in incidence of primary tumours of lung in mice exposed to dust obtained from tarred roads, 287

CLARKE, L. P., see *Findlay, G. M.*

COLLIP, J. B., PUGSLEY, L. I., SELYE, H., and THOMSON, D. L., observations concerning the mechanism of parathyroid hormone action, 335

CRAIGIE, J., and WISHART, F. O., the agglutinogens of a strain of vaccinia elementary bodies, 390

DELAFIELD, M. E., blood-sugar changes and toxic effects produced in rabbits by certain fractions derived from *Bact. aertrycke*, 130

DEVEGNEY, F. E. C., a new agent for stimulating the metabolism: dinitro-O-cyclopentylphenol (Prep. 2769/1) and its action on the basal metabolism, 360

DE WESSELOW, O. L. V. S., and GRIFFITHS, W. J., on the question of pressor bodies in the blood of hypertensive subjects, 45

DUNCAN, J. T., optimal agglutination: the significance of the different ratios of serum to bacteria, 23

FELIX, A., BHATNAGAR, S. S., and PITT, R. MARGARET, observations on the properties of the Vi antigen of *B. typhosus*, 346

FERRY, J. D., see *Burnet, F. M.*

FINDLAY, G. M., and BROWN, H. C., the relation of the electric charge of the red cells to phagocytosis in avian malaria, 148

——— and CLARKE, L. P., the experimental production of mumps in monkeys, 309

FRASER, D. T., see *Silverthorne, N.*

GALLOWAY, I. A., the "fixed" virus of rabies: the antigenic value of the virus inactivated by the photodynamic action of methylene-blue and proflavine, 97

GOUGH, G. A. C., the purification of tuberculin, 237

GREEN, H. N., changes in the esterase and fat content of the serum induced by cancer and cancer-producing agents, 1
GRIFFITHS, W. J., see *de Wesselow, O. L. V. S.*

HAM, A. W., and MURRAY, L. D., hypervitaminosis D rickets: the action of vitamin D, 228
HARLEY, D., the immunizing action of extracts of pneumococci (Types 1 and 2) in mice and rabbits, 161
HENDERSON, D. W., experiments with the "O" antigen of *Clostridium œdematis maligni* (*Vibrion septique*), 166

KENDAL, L. P., see *Berenblum, I.*
KLIGLER, I. J., and ASCHNER, M., immunization of animals with formolized tissue cultures of *Rickettsia* from European and Mediterranean typhus, 337
——— and OLITZKI, L., studies on the protein-free suspensions of viruses. V, Further observations on the nature and antigenic properties of a highly purified phage, 14

LOEWENTHAL, H., type-specific and group-specific sera against streptococci, 298

MCEUEN, C. S., and THOMSON, D. L., the growth of the Walker rat tumour in young and old animals, 224
——— SELYE, H., and THOMSON, D. L., the effect of malignant tumours on the hypophysis, 221
MACLEAN, D. L., and BEST, C. H., choline and liver fat, 193
MARTIN, A. R., the toxicity for mice of certain fractions isolated from *Bact. aertrycke*, 137
MILLER, E. W., see *Pybus, F. C.*
MOTTRAM, J. C., some effects of cancer-producing agents on the chromosomes, 71
MURRAY, L. D., see *Ham, A. W.*

OLITZKI, L., see *Kligler, I. J.*
O'MEARA, R. A. Q., routine preparation of scarlet fever toxin of high value, 295
ORR, J. W., the influence of ischæmia on the development of tumours, 73

PESKETT, G. L., the determination of blood-urea, 306
PITT, R. MARGARET, see *Felix, A.*
PLUMMER, HELEN, the accuracy of the skin test in chinchilla rabbits in the determination of the strengths of streptococcal toxins and antitoxins, 80
PUGSLEY, L. I., see *Collip, J. B.*
PYBUS, F. C., an attempt to produce immunity to induced tumours in mice, 89
——— and MILLER, E. W., on the existence of growth-promoting and growth-inhibiting properties (*in vitro*) of the sera and plasma of mice naturally immune, or with acquired resistance to a tar-sarcoma, 207

RAISTRICK, H., and TOPLEY, W. W. C., immunizing fractions isolated from *Bact. aertrycke*, 113

SABIN, A. B., studies on the B virus. I, the immunological identity of a virus isolated from a human case of ascending myelitis associated with visceral necrosis, 248
——— studies on the B virus. II, properties of the virus and pathogenesis of the experimental disease in rabbits, 268
——— studies on the B virus. III, the experimental disease in *Macacus rhesus* monkeys, 321
——— the immunological relationships of pseudorabies (infectious bulbar paralysis, mad itch), 372
SALAMAN, M. H., the flocculable substance of vaccinia: the effect on its antigenic properties of adsorption on collodion particles, 381
SCHÜTZE, H., the envelope antigen of *B. pestis* and its antibody, 200
SELYE, H., see *Collip, J. B.*, and *McEuen, C. S.*
SILVERTHORNE, N., and FRASER, D. T., the action of human blood on the meningococcus, 362

THOMSON, D. L., see *Collip, J. B.*, and *McEuen, C. S.*
TOPLEY, W. W. C., see *Raistrick, H.*

WHITEHEAD, R., the cortical lipoid of the suprarenal in mice (1) with infectious ectromelia, (2) in starvation, (3) exposed to heat, and (4) fed on cholesterol, 279
WILLIS, R. A., an experimental study of the possible influence of injury in the genesis of tumours of the gonads, 234
WISHART, F. O., see *Craigie, J.*

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CONTENTS.

SABIN, ALBERT B.—STUDIES ON THE B. VIRUS. III: THE EXPERIMENTAL DISEASE IN *MACACUS RHESUS* MONKEYS 321

COLLIP, J. B., PUGSLEY, L. I., SELYE, H., AND THOMSON, D. L.—OBSERVATIONS CONCERNING THE MECHANISM OF PARATHYROID HORMONE ACTION . 335

KLIGLER, I. J., AND ASCHNER, M.—IMMUNIZATION OF ANIMALS WITH FORMOLIZED TISSUE CULTURES OF *RICKETTSIA* FROM EUROPEAN AND MEDITERRANEAN TYPHUS 337

FELIX, A., BHATNAGAR, S. S., AND PITT, R. MARGARET.—OBSERVATIONS ON THE PROPERTIES OF THE VI ANTIGEN OF *B. TYPHOSUS* 346

BURNETT, F. M.—ANTIGENIC DIFFERENCES BETWEEN RELATED BACTERIAL STRAINS: A CRITICISM OF THE MOSAIC HYPOTHESIS 354

DEVEGNEY, F. E. C.—A NEW AGENT FOR STIMULATING THE METABOLISM: DINITRO-O-CYCLOPENTYLPHENOL (PREP. 2769/1) AND ITS ACTION ON THE BASAL METABOLIC RATE 360

SILVERTHORNE, NELLES, AND FRASER, DONALD T.—THE ACTION OF HUMAN BLOOD ON THE MENINGOCOCCUS 362

BERENBLUM, I., AND KENDAL, L. P.—THE PRODUCTION OF TUMOURS IN THE FOWL WITH A COLLOIDAL SOLUTION OF 1 : 2 : 5 : 6-DIBENZANTHRACENE 366

SABIN, ALBERT B.—THE IMMUNOLOGICAL RELATIONSHIPS OF PSEUDORABIES (INFECTIOUS BULBAR PARALYSIS, MAD ITCH) 372

SALAMAN, M. H.—THE FLOCCULABLE SUBSTANCE OF VACCINIA: THE EFFECT ON ITS ANTIGENIC PROPERTIES OF ADSORPTION ON COLLODION PARTICLES 381

CRAIGIE, JAMES, AND WISHART, F. O.—THE AGGLUTINOGENS OF A STRAIN OF VACCINIA ELEMENTARY BODIES 390

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